Vol. XXXVIII September, 1949 No. 9

SCIENTIFIC EDITION

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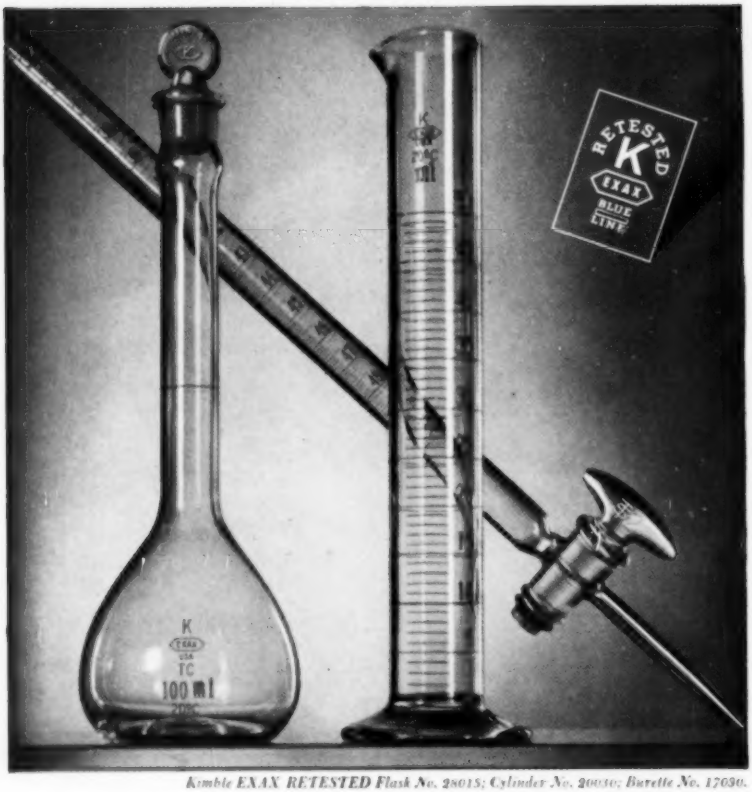

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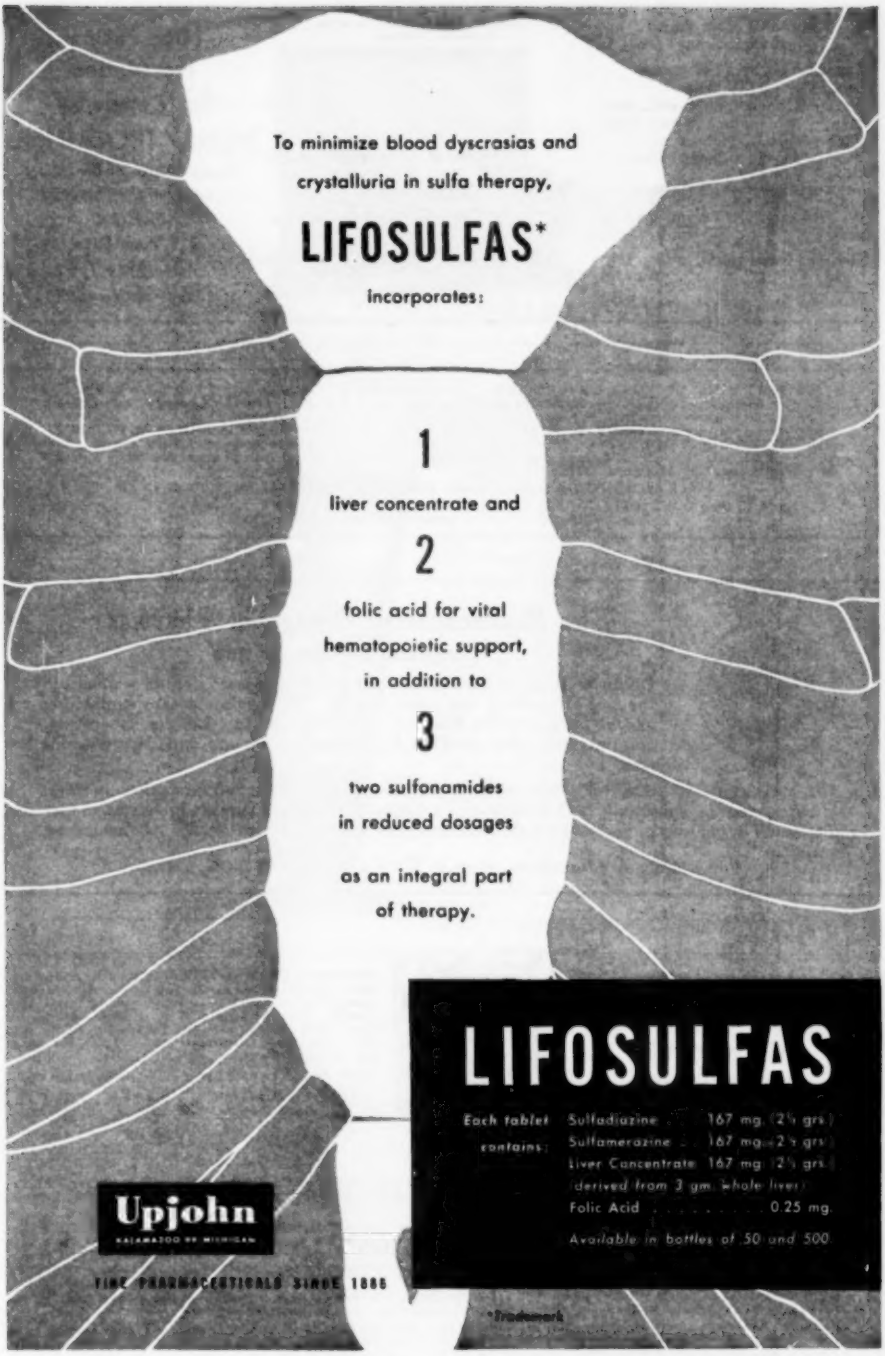
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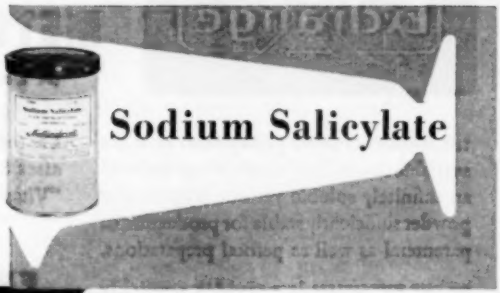

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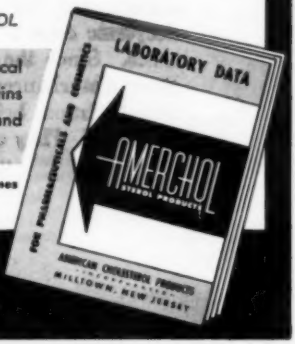

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VOLUME XXXVIII | *SEPTEMBER, 1949* | NUMBER 9 | CONSECUTIVE NO. 18

The Hydrogen Ion Concentration and Osmotic Properties of Lacrimal Fluid*

By HARRY W. HIND and FRANK M. GOYAN†

The *p*H of tears is approximately 7.4. Further study reveals the fact that the *p*H of tears increases very rapidly to a value of 8.2 or higher on standing under conditions which permit loss of carbon dioxide. There is now general agreement that tears are isotonic with 0.9 per cent sodium chloride solution.

THERE IS considerable controversy in the literature concerning the physicochemical properties of lacrimal fluid. The hydrogen-ion concentration, or *p*H, as normally determined, colorimetrically or electrometrically, has been reported to have values ranging from 6.3 to 8.6 (1–7). The controversy seems to hinge around the value 7.4 and more alkaline values of approximately 8.2. Extended experimental studies over a period of several years convinced the present authors that the value of 7.4 represents the approximate *p*H of lacrimal fluid. Loss of carbon dioxide readily accounts for higher *p*H values.

There is also confusion in the literature concerning osmotic properties of lacrimal fluid. A value of 1.4 per cent is often given for the concentration of a sodium chloride solution isotonic with tears. This value is not in agreement with any physicochemical measurement; in fact, it stems from early work based upon a subjective approach. The value of 1.4 was traced back to Massart (1889) by Krogh (8) who reports that Massart's experiments consisted in instilling drops of different solutions of sodium chloride into the eyes of two subjects. On the basis of the subjective judgment of these two subjects, a solution concentration of 1.4 per cent was selected as most suitable. This value has been carried through the pharmaceutical literature from this early date without checking the original source. As early as 1943 it was apparent to the authors (9, 10) that the value of 1.4 was incorrect; that solutions isotonic to blood serum seemed to approximate the osmotic effect of tears. However, it remained for Krogh (8) to establish this value by vapor-pressure measurements based upon the thermoelectric method (11). Tentative

* Received April 29, 1949, from the Barnes-Hind Laboratories, Inc., 430 Post St., San Francisco, Calif.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† University of California Medical Center, College of Pharmacy, San Francisco, Calif.

measurements of a similar nature made by the authors are in substantial agreement.[1]

*p*H DETERMINATIONS

It is well established in the ophthalmological literature that carbon dioxide is expired from the normal human eye. Hind (10) has shown that the expiration of carbon dioxide is a significant factor in the design of contact lens solutions, indicating that a successful technique for the determination of *p*H of lacrimal fluid must include means for the prevention of loss of carbon dioxide.

Collection of Tear Sample.—The lower eyelid is pulled forward in order to form a well-like pocket. Two to three drops of boiled, cool distilled water are instilled; the irritation caused by the distilled water produces a copious flow of tears. As soon as sufficient lacrimal fluid is available to fill the electrode chamber, the sample of tear is drawn directly into the electrode chamber, or into a medicine dropper for transfer to a "one-drop" electrode. This method of tear collection is used for *p*H determination only.

Electrode Systems.—A Beckman "one-drop" electrode, Catalogue No. 289K, and a Coleman Micro-Chamber Electrode for collection of blood samples (Catalogue No. 3-200) were employed with a potassium chloride bridge extending to a saturated calomel electrode. Measurements were made with a Beckman Laboratory Model *p*H meter, and also with the *p*H meter described by Goyan, Barnes, and Hind (12). The MacInnes and Dole membrane-type glass electrode (13) was used for a few measurements in vessels somewhat similar to the commercial electrodes described above. The Beckman electrode is a glass electrode shaped like an open cup partially covered with the potassium chloride bridge. When using this electrode, it is necessary to work rapidly, taking the initial reading within approximately five seconds after removing the tear sample. The "blood-sample" electrode is an enclosed glass electrode. When using this electrode great speed is not so critical, although there is a marked tendency for the pH of tears to increase gradually on standing.

Results.—It has been established experimentally by Hosford and Hicks (3) and confirmed by Feldman (1) that the *p*H of a tear sample is not materially changed by considerable dilution. This evidence, combined with the fact that the volume of distilled water required to be instilled into the eye is relatively small, suggested the application of the above technique in routine fitting of the contact lens solutions (10). These routine *p*H measurements were made over a period of years. One series of 77 normal individuals seeking to use contact lens solutions donated tear samples ranging in *p*H from 7.3 to 7.7, the average of the 77 *p*H values being 7.47. A few similar measurements made at a later date show a steady increase in *p*H on standing up to a value as high as 8.5; the rate of increase being roughly determined by the ease of escape of carbon dioxide as shown in Fig. 1. Although the routine measurements were made rapidly, the observed increase in

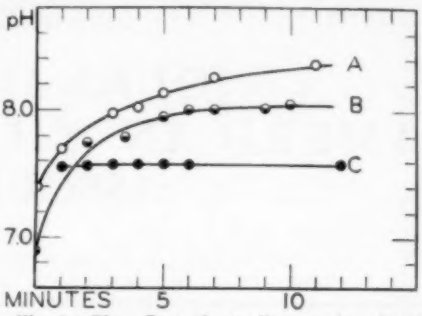

Fig. 1.—The effect of standing on the *p*H of lacrimal fluid. Curve *A* represents a sample exposed to air on a glass membrane (inverted MacInnes and Dole type glass electrode), curve *B* represents a similar sample in a cup-type glass electrode, and curve *C* shows the same type of measurement in a completely enclosed electrode chamber using the MacInnes and Dole electrode.

*p*H on standing indicates that the average for the 77 measurements would tend to be slightly high, thus justifying the statement that the approximate *p*H of lacrimal fluid is 7.4. It is difficult to establish the absolute *p*H of lacrimal fluid with high accuracy because of the technical difficulties arising from the fact that the *p*H *in vitro* is evidently changing with time.

DISCUSSION

The above findings are reported to confirm the results reported by others (1, 3) that normal lacrimal fluid and blood have about the same *p*H. *In vitro*, and perhaps in certain pathological conditions (6), loss of CO_2 may account for higher *p*H values. The authors became interested in roughly checking this point by the use of an indicator paper technique described by Goyan and Coutsouris (14) in which bromothymol blue paper moistened with fresh tears is compared with freshly made standards. It is very easy to demonstrate by this means the increase in *p*H from approximately 7.4, when the wet paper is in the eye, to a more alkaline value after exposure to air. It is of interest that the color change may be reversed merely by blowing the breath, which is rich in carbon dioxide, upon the paper. Electrometric determinations further confirm the conclusion that loss of carbon dioxide is responsible for high *p*H values reported for lacrimal fluid. The results of these determinations are shown graphically in Fig. 2 where *p*H is plotted as a function of the time in order to demonstrate the effect of changes which influence the rate of loss or gain of CO_2.

SUMMARY

The *p*H of tears is approximately 7.4. Further study reveals the fact that the *p*H of tears increases very rapidly to a value of 8.2 or higher on standing under conditions which permit loss of carbon dioxide. There is now general agreement that tears are isotonic with 0.9 per cent sodium chloride solution.

[1] Undiluted lacrimal fluid in contact with thermocouple junctions is used in all tonicity measurements of this type.

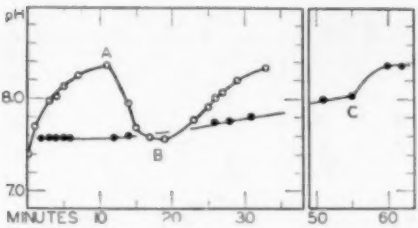

Fig. 2.—Effect of altering conditions on the pH of lacrimal fluid. Open circles represent measurements made with an open membrane glass electrode (inverted MacInnes and Dole type). At point *A* a thin plastic film was used to enclose exhaled air (rich in CO_2) above and around the otherwise open sample. At point *B* the plastic film was removed and the sample was again freely exposed to the air of the room. Dark circles represent measurements made with a completely enclosed electrode chamber. At point *B* the seal was broken, and at point *C* nitrogen (free of CO_2) was bubbled through the sample.

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Botanical and Phytochemical Studies in *Hyoscyamus Muticus*, L. I.*

By Z. F. AHMED† and I. R. FAHMY‡

Alkaloid formation during the development of *Hyoscyamus muticus* has been followed and the effect of certain environmental conditions upon its growth and alkaloidal content have been determined and are reported in this paper.

THE PLANT *Hyoscyamus muticus* L. was one of the sacred plants used by the ancient Egyptians. At the present time, it ranks among the most important medicinal plants of the Egyptian market owing to its high alkaloidal content (1-4). It is generally collected by Arab shepherds from wild growing plants which are found scattered here and there in the Egyptian deserts. The plant has been cultivated in certain limited areas of Upper Egypt for exportation. Although this plant was known long ago, specific phytochemical and ecological studies of it are lacking. Therefore, it was deemed of interest to follow the alkaloid formation through the development of the plant and to determine the effect of certain environmental conditions on its growth and alkaloidal content. For this purpose experiments were carried out at the experimental station attached to the Pharmacognosy Department, Fouad I. University, Cairo, Egypt, and the results obtained are presented in this paper.

EXPERIMENTAL

Microdetection and Localization of Hyoscyamine

Rosenthaler (5) found that Wagner's reagent gave with hyoscyamine a characteristic precipitate in a fairly large dilution. The reaction was studied and the reagent was successfully applied to the detection and localization of the alkaloid hyoscyamine in *Hyoscyamus muticus* L., since hyoscyamine is the chief alkaloid found in this plant.

The Use of Wagner's Reagent for the Microdetection of Hyoscyamine.—The sensitivity of Wagner's reagent toward hyoscyamine was first found out by preparing a 1% *w/v* aqueous solution of hyoscyamine (Merck) with the aid of dilute acetic acid. This solution was diluted successively to such an extent that one drop of it when mixed with one drop of the reagent failed to produce any precipitate

* Received Nov. 12, 1947, from the Pharmacognosy Department, Fouad I. University, Cairo, Egypt.
† Lecturer of Pharmacognosy, Faculty of Medicine, Fouad I. University.
‡ Professor of Pharmacognosy, Faculty of Medicine, Fouad I. University.

as seen by the naked eye. This was obtained when the solution was diluted to the extent of 1:25,000.

When one drop of a solution of hyoscyamine 1:25,000 was treated with an equal drop of Wagner's reagent and the formation of the precipitate was followed under the microscope, it appeared to start as very minute dots which increased in number and size, rapidly becoming rounded. These rounded crystals increased in size until they attained 11 to 15 μ in diameter when they acquired the shape of flying birds or crosses with thickened centers and tapering arms. Sometimes needle-shaped crystals were seen as well.

More concentrated solutions of hyoscyamine gave with the reagent immediate copious precipitate of large needle-shaped crystals which attained in some cases 40 μ in length.

In less concentrated solutions of hyoscyamine, the precipitate was formed gradually, and at the same time the crystals became smaller in size and fewer in number.

At a concentration of 1:50,000 of hyoscyamine only a few crystals exhibited the shape of flying birds, while most of them showed a great variation in shape and reaching 7 to 11 μ in diameter.

At a concentration of 1:100,000 of hyoscyamine, the precipitate exhibited a great number of small globular, subglobular, and dumbbell-shaped structures attaining 3 to 7 μ in diameter.

At a concentration less than 1:100,000 of hyoscyamine the precipitate exhibited a great number of minute oily droplets which decreased in number and size on further dilution of the hyoscyamine solution, until no more precipitate was perceptible in a dilution of 1:125,000 at a magnification of 300.

The Use of Wagner's Reagent in the Localization of Hyoscyamine in the Tissues of *Hyoscyamus muticus L.*—To follow the formation and localization of hyoscyamine in the different tissues of the *Hyoscyamus muticus* L., surface preparations and sections were made successively in the seeds, seedlings, and the plant at different stages of its growth until maturity.

These preparations were made in the fresh material and treated with Wagner's reagent which gave, with hyoscyamine in dilution up to 0.002%, characteristic crystals of hyoscyamine iodide having the shape of flying birds (Fig. 1). The localization of the hyoscyamine in the tissues was followed by placing the preparation on a microscopic slide and irrigating it with the reagent and observing the gradual appearance of the crystals within the different tissues (Fig. 2). Another relatively thick preparation of the same tissue was dipped in the reagent for a few minutes, washed with water, and examined under a microscope to assure the localization of the alkaloid in the intact tissues.

Surface preparations of the seed coat treated with Wagner's reagent showed typical hyoscyamine iodide crystals inside the cells, while the reagent failed to produce any precipitate within the endosperm and embryo cells.

On the first day of germination of the seeds only a few cells of the seed coat responded to the reaction and it failed completely in the succeeding days.

Up to the thirteenth day the reagent failed to give a precipitate with the tissues of the different organs of the seedling.

Fig. 1.—A photomicrograph of the hyoscyamine iodide crystals showing at the same time incomplete forms, × 400.

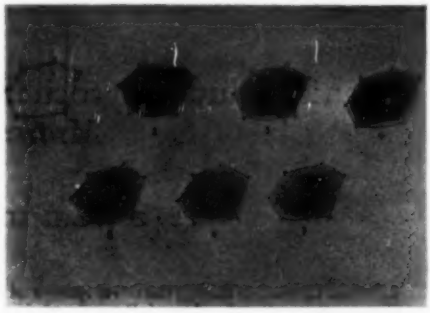

Fig. 2.—A photograph of freehand drawing showing, from *1* to *7*, the steps of formation of hyoscyamine iodide precipitate inside the plant cell.

1, the cell before treatment with the reagent; *2*, the yellowish brown color of the reagent; *3*, the violetish tinge in the middle of the cell; *4*, the appearance of very minute violet dots; *5*, the increase in the size and number of the dots; *6*, the beginning of the formation of the typical crystals of the precipitate; *7*, the perfect flying bird-shaped crystals of the precipitate.

On the fourteenth day the parenchyma of the central region of the cotyledons gave a typical precipitate with the reagent, while the tissues of other organs of the seedling did not respond to the test.

On the fifteenth day the alkaloidal reaction became less perceptible until it entirely disappeared from the cotyledons on the eighteenth day and then started to appear again in the parenchyma of the central region of the leaf and petiole of the second pair, as well as in the basal cells of the large hairs of the young third pair of leaves.

On the twenty-first day the intensity of the test was more pronounced in these tissues and at the same time the epidermal cells and the spongy parenchyma of the leaf, as well as the cortical parenchyma of the

hypocotyl, started to respond to the test, which gradually intensified in these tissues. The alkaloid tended to localize in the epidermal tissues up to the thirty-third day, when it reached its maximum concentration in the epidermis of the petioles and leaves. At this stage the test failed in the tissues of the second pair of leaves and, at the same time, it started to appear in the parenchyma cells of the cortex, pith, and to a less extent in the epidermis of the young, newly formed stems, with a tendency to concentrate in the peripheral zones of these tissues. As the plant developed, the alkaloid tended to accumulate in the parenchyma tissues of both the pith and cortex, although the young developing parts of the plant always had their alkaloid more concentrated in their peripheral tissues.

In all the stages of growth of the plant, the palisade tissue of the leaf and the conducting tissues of the plant failed to give the test with Wagner's reagent. It was also noticed that the localization reaction was more intense in the young developing organs, while it failed in the withering leaves.

Up to the fortieth day of the growth of the plant, the root tissues did not respond to the reagent. However, on the forty-fifth day the exodermal cells and the cells of the outer cortical parenchyma responded to the reagent and continued to do so until the seventy-fifth day, when the reaction failed gradually, owing to the exfoliation of the cortical tissues by the formation of cork cambium.

In the flowering plant, the pith and the cortical tissues responded strongly to Wagner's reagent, while the response of the epidermis to the reagent came next, and lastly the response of phloem parenchyma and the spongy parenchyma of the blade.

The Alkaloidal Content.—To determine the alkaloidal content at the different stages of the growth of the plant, a sample of viable seeds, previously subjected to complete analysis, was sown in pots and in a limited area of a sandy-loam soil.

Representative samples of the seedlings were assayed every twenty-four hours for the first week, after which the overground and underground parts of the plants were assayed separately every twenty-four hours for two months, then every week until the flowering of the plant.

Since the weight of the individual plant varied from a small fraction of a gram in the early stages of growth to even more than a kilogram in the flowering stage, the number of the plants in the samples analyzed varied accordingly from thousands to few individuals, respectively.

The moisture content was determined by drying a representative sample immediately after collection in an oven at 100° to a constant weight. The dried material was then ignited and the percentage of ash calculated on the material dried at 100°.

The methodical collection of thousands of intact seedlings during the underground stages of growth of the plant needed the patient collaboration of several persons.

The mean values of the results obtained are given in Table I.

From Table I it is noticed that, on germination, a gradual decrease in the alkaloid takes place in the seedlings until the appearance of the cotyledons over the ground, when the alkaloid starts to increase, attaining a maximum about the fourteenth day. This is followed by a gradual decrease until about the fifth week when the alkaloid remains, with a slight decrease, almost constant up to the flowering stage, when it rises again.

On fruiting, the alkaloidal content is reduced to less than half that in the flowering plants.

No alkaloid could be detected in the roots until about the fifth week of their growth. Then, as seen in Table I, it increased gradually for five weeks, at the end of which time it begins to drop gradually until the flowering stage. At this stage the alkaloids occur only in traces in the roots. At the fruiting stage the percentage of alkaloids in the roots is almost equal to that in the overground parts of the plant.

The method of assay used for the determination of alkaloids in the above experiments is that of the first edition of the Egyptian Pharmacopœia, which is essentially the same as that of U. S. P. XIII.

Moisture.—The results given in Table I show that the moisture content increases gradually from about 8% in the seeds to about 94% at the end of the second week of growth of the seedling, and then remains almost constant in the overground parts of the plant up to the flowering stage. On fruiting, the moisture drops to about 8% in the overground parts of the plant.

The moisture in the roots is shown to be about 70% at the end of the fifth week of growth, when it increases gradually to about 90% during the next two weeks. It starts to drop gradually until the flowering stage, when it becomes about 85%. On fruiting, the moisture in the root drops only slightly.

Ash.—The results given in Table I show that the amount of ash increases gradually from about 4% in the seed to about 20% in the overground parts of the dry, mature plant. The ash in the roots is about 10% at the end of the fifth week of growth and increases gradually to about 20% in the mature fruiting plant.

Distribution of the Alkaloids in the Flowering Plant.—To find out the distribution of the alkaloid in the different parts of the plant, the uppermost parts, the central parts, and the lowermost parts of three representative samples of well-developed flowering plants, attaining more than a meter in height, were separated. From each of these parts, the leaves and stems were separated. The separated organs of each part were analyzed to determine their alkaloidal and moisture content. The results obtained, calculated on the material dried at 100°, are given in Table II.

From Table II one may conclude that the actively developing uppermost parts of the plant contain the highest percentage of alkaloid, then follow the central and, last, the lowermost parts which contain the lowest percentage of alkaloid. The leaves, as a whole, contain a higher percentage of alkaloid than the stems.

In regard to the moisture content, it is noticed that it increases, going upward in case of the stems, and downward in case of the leaves.

The Alkaloidal Content of the Different Organs of the Plant.—To find out the alkaloidal content of the different organs of the fresh plant, a truckload of the fresh, wild flowering plant and the equivalent of six bushels of flowers collected from the Suez Road area were immediately transferred to the laboratory in Cairo, where the plants were separated into their

TABLE I.—PERCENTAGE OF TOTAL ALKALOIDS, MOISTURE, AND ASH IN THE OVERGROUND AND UNDERGROUND PARTS OF THE PLANT, AT DIFFERENT STAGES OF ITS GROWTH

Age of Sample in Days	Overground Part of Plant			Underground Part of Plant		
	Total Alkaloids, %	Moisture, %	Ash, %	Total Alkaloids, %	Moisture, %	Ash, %
Seeds	0.700	8.0	3.92	0	..	..
1	0.301	12.4	4.36	0	..	..
2	0.190	31.5	6.26	0	..	..
3	0.100	42.0	9.94	0	..	..
4	0.200	45.0	10.20	0	..	..
5	0.250	50.0	10.11	0	..	..
6	0.300	60.0	5.00	0	..	..
7	0.320	63.0	8.00	0	..	..
8	0.320	67.0	10.00	0	..	..
9	0.860	75.0	10.30	0	..	..
10	1.040	85.0	11.22	0	..	..
11	1.500	90.5	11.65	0	..	..
12	2.400	92.5	12.36	0	..	..
13	2.700	93.0	13.25	0	..	..
14	3.090	93.0	15.20	0	..	..
15	2.760	93.2	15.28	0	..	..
16	2.650	93.7	16.35	0	..	..
17	2.500	94.0	16.92	0	..	..
18	2.300	94.0	16.85	0	..	..
19	2.000	94.0	16.85	0	..	..
20	1.900	94.5	17.64	0	..	..
21	1.900	94.5	17.56	0	..	..
22	1.900	94.5	17.57	0	..	..
23	1.900	94.5	17.52	0	..	..
24	1.900	95.0	17.50	0	..	..
25	1.900	95.0	17.45	0	..	..
26	1.800	95.0	17.78	0	..	..
27	1.560	95.0	18.02	0	..	..
28	1.300	94.5	18.21	0	..	..
29	1.250	95.0	18.64	0	..	..
30	1.250	95.0	18.77	0	..	..
35	1.200	95.3	19.76	0.080	70.0	10.0
41	0.960	95.5	19.80	0.173	75.0	10.3
48	1.050	95.2	19.80	0.320	82.0	12.2
55	1.000	95.0	20.00	0.567	90.0	11.9
62	1.000	95.0	21.00	0.876	89.0	12.0
69	1.000	94.5	22.00	0.950	89.0	12.0
76	1.000	94.5	22.50	0.640	89.0	12.0
83	1.000	94.0	23.00	0.590	88.8	12.0
90	1.100	93.5	23.50	0.503	88.5	13.0
Flower	1.200	93.5	24.00	0.320	85.0	18.0
Fruit	0.500	8.0	20.00	0.450	82.0	20.0

TABLE II.—THE DISTRIBUTION OF THE ALKALOID IN THE PLANT

Sample No.	Uppermost Leaves		Central Leaves		Lowermost Leaves	
	Alkaloids, %	Moisture, %	Alkaloids, %	Moisture, %	Alkaloids, %	Moisture, %
1	1.94	87.0	1.65	93.0	0.6	94.8
2	2.08	88.0	1.87	92.0	0.7	94.4
3	2.31	90.0	1.83	91.5	0.77	95.0
Mean	2.11	88.5	1.78	92.25	0.735	94.7

Sample No.	Uppermost Part of Stems		Central Part of Stems		Lowermost Part of Stems	
	Alkaloids, %	Moisture, %	Alkaloids, %	Moisture, %	Alkaloids, %	Moisture, %
1	1.03	95.1	0.602	94.0	0.411	93.0
2	1.28	95.5	0.710	94.4	0.420	93.3
3	1.13	95.0	0.747	94.2	0.443	93.5
Mean	1.14	95.1	0.643	94.3	0.432	93.3

different organs, namely the flowers, the leaves, the stems, and the roots. Most of the leaves were further separated by macrodissection into their petioles, blades, and midribs, while most of the flowers were separated similarly into their calyces, corollas, anthers, filaments, styles with stigmas, and ovaries with immature ovules.

For each of these parts, sufficient amounts of the fresh material were available for moisture and ash determination, and of the air-dried material for duplicate alkaloidal assay.

The mean values of the results obtained, calculated on the material dried at 100°, are given in Table III.

TABLE III.—THE ALKALOIDAL CONTENT OF THE DIFFERENT ORGANS OF THE PLANT

Plant Organ	Alkaloids, %	Moisture, %	Ash, %
I—Flower	2.0	88	20
a—Calyx	2.5	89	22
b—Corolla	2.2	90	17
c—Anthers	2.11	81.3	5
d—Filaments	1.751	80.3	7
e—Styles with stigmas	1.135	80	7
f—Ovaries with immature ovules	0.95	43	19
II—Leaves	1.7	94.8	25
a—Petioles	1.76	94.5	20
b—Blades	1.6	94.57	22
c—Midribs	1.36	93.5	18
III—Stems	0.51	92	23
IV—Root	0.3	82	21

From Table IV one may conclude that pigmented organs contain about double the amount of alkaloids as the respective nonpigmented organs, and accordingly, plants with pigmented organs are richer in alkaloids than those with nonpigmented organs.

SUMMARY

The study of the precipitation of hyoscyamine with Wagner's reagent furnished a good means for the microdetection and localization of the alkaloids in the tissues of *Hyoscyamus muticus* L.

The highest alkaloidal percentage is found in the floral parts, followed by the leaves, then the stems, and finally the roots of the plant, which contain the lowest percentage.

The highest yield of plant material and highest alkaloidal content are obtained from the over-ground parts of the plant at the flowering stage.

The variation in the color of the pigment is most probably due to the change in the reaction of the cell-sap, which inclines toward acidity as the plant develops. A possible parallelism between the appearance of the anthocyanin pigment and the formation of the alkaloid might exist.

TABLE IV

Sample No	Corollas		Petioles		Stems	
	Pigmented	Nonpigmented Alkaloids, %	Pigmented	Nonpigmented Alkaloids, %	Pigmented	Nonpigmented Alkaloids, %
1	1.99	0.954	1.28	0.97	0.91	0.56
2	1.93	0.933	1.08	0.76	0.87	0.49
3	1.73	0.867	1.05	0.72	0.77	0.48
Mean	1.883	0.918	1.103	0.816	0.85	0.51

The Coloring Matter.—The anthocyanin pigment was found to exhibit a series of color changes through the different stages of growth of the plant. This was most probably due to variation in the reaction of the cell-sap which inclined toward acidity as the plant developed. It was marine-blue in the young plantlet when the *p*H of the cell-sap was about 7.37; purplish blue in older plants when the *p*H was about 6.47, and purplish red in the flowering plants when the *p*H was about 5.68.

Organs displaying anthocyanin pigment in their tissues were found to contain a higher percentage of alkaloid than the same when devoid of it.

In order to verify this fact, duplicate analyses of different representative samples of corollas, petioles, and stems of the fresh flowering plants containing the pigment, and similar organ of plants from the same lots free from the pigment, were made.

The mean values of the results obtained, calculated on the material dried at 100°, are given in Table IV.

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The Effect of Environment on the Growth and Alkaloidal Content of *Hyoscyamus Muticus* L. II.*

By Z. F. AHMED† and I. R. FAHMY‡

The effects of soil, irrigation, fertilizing, form of cultivation, and other environmental factors on *Hyoscyamus muticus* during cultivation have been determined. The effect of the factors studied upon the yield of alkaloids from the plant is particularly emphasized.

AMONG the main factors governing the growth of plants and consequently their crops and constituents are: soil, irrigation, fertilizing, season of cultivation, sunshine, and disease (1). The effect of these factors on *Hyoscyamus muticus* L. during cultivation, as to its crop and alkaloidal content, was studied.

Different experiments were carried out at the experimental station attached to the Pharmacognosy Department, Faculty of Medicine, Cairo, Egypt. The observations and the results obtained are presented in this paper.

EXPERIMENTAL

Soil and Irrigation.—Five types of soils (2), the true sand, the true clay, the sand, the loam, and the clay soils were used.

Attempts to grow or transplant the plant in the true sand and the true clay at different seasons were unsuccessful.

Preliminary experiments showed that excessive irrigation was detrimental to the life of the plant and to its alkaloidal content, but the plant survived drought after germination for a long period of time.

Plants grown in comparatively dry soil yield a higher alkaloidal percentage than those grown in damp places.

To study the effect of soil and water on the vegetative growth as well as on the alkaloidal content of the plant, three separated equal areas of each of the three types of soils mentioned, namely the sand, the loam, and the clay, were prepared for cultivation.

The same amounts of a viable sample of seeds were sown broadcast in each of these areas on the same day.

Water for all areas was given slowly so as to cover the earth by a layer attaining 7 cm. in height in the first irrigation and 4 cm. only in the following irrigations.

The number of irrigations varied consistently in each of the three areas of each type of soil. Corresponding areas were irrigated simultaneously.

The flowering plants of each area were collected separately, weighed fresh, and their alkaloidal and moisture contents determined. The results obtained are given in Table I.

From Table I one may conclude that the plant can grow in the sand and clay soils but flourishes best in the loamy soil.

The alkaloidal percentage invariably decreases with the increase of water supply.

TABLE I.—SHOWING THE NUMBER OF IRRIGATIONS, THE ALKALOIDAL PERCENTAGE CALCULATED ON ANHYDROUS MATERIAL, THE MOISTURE CONTENT AND THE APPROXIMATE YIELD OF THE AIR-DRIED MATERIAL EXPRESSED IN GM./SQ. METER FOR EACH SOIL SEPARATELY

Soil Plots	No. Irrigations	Total Alkaloids, %	Moisture, %	Approximate Vegetative Yield in Gm./Sq. Meter on Air-Dried Material
1—Clay 1	12	0.80	93.8	1380
2—Clay 2	8	1.00	93.6	1325
3—Clay 3	4	1.00	93.5	1125
4—Loam 1	12	0.86	93.6	1650
5—Loam 2	8	1.10	93.4	1500
6—Loam 3	4	1.20	93.2	1000
7—Sand 1	12	0.88	93.6	1550
8—Sand 2	8	1.15	93.4	1450
9—Sand 3	4	1.30	93.2	1000

Fertilizing.—It has been stated that fertilizing increases the alkaloidal content in solanaceous plants (3). Two nitrogenous fertilizers, the farmyard organic manure containing about 0.6% available nitrogen, and the Chilean nitrate, containing about 15.5% available nitrogen, were used to determine their effects on the vegetative growth and the alkaloidal content of the plant. Various quantities of each fertilizer were mixed in separate plots of sandy-loam soil, keeping some plots without any fertilizer for control. The assigned quantity for each plot was administered in two equal portions, one after four weeks, and the other after six weeks of plant growth. The mean values of the results obtained are given in Table II.

From Table II one may conclude that the application of nitrogenous fertilizers, especially the inorganic type, increases the alkaloidal content and the vegetative yield of the cultivated plant. It is also noticed that the alkaloidal percentage in the root is almost constant and is unaffected by fertilizing.

Shade.—Plants grown in absolute shade compared to those grown in the sun showed: (*a*) The vegetative organs were very weakly developed. (*b*) The covering hairs were few in number, very rarely branched. (*c*) The calcium oxalate crystals were very few, rarely exceeding 26 μ in diameter. (*d*) The blue anthocyanin pigment was not produced. (*e*) The alkaloidal content was very poor, being one-

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TABLE II.—SHOWING THE VEGETATIVE YIELD IN GM./SQ. M. OF THE AIR-DRIED MATERIAL, THE ALKALOIDAL AND MOISTURE CONTENTS BOTH OF THE OVERGROUND AND UNDERGROUND PARTS OF THE FLOWERING PLANT GIVEN DIFFERENT QUANTITIES OF MANURE

Plot No.	Quality and Quantity of Fertilizer Administered	Approx. Vegetative Yield in Gm./Sq. M. Air-Dried Material	Overground Part of Plant		Underground Part of Plant	
			Total Alkaloids, %	Total Moisture, %	Total Alkaloids, %	Total Moisture, %
1	Farmyard manure 25 L./sq. m.	1550	1.33	93	0.34	82.0
2	Chilean nitrate 12 Gm./sq. m.	1430	1.272	93	0.325	83.5
3	Chilean nitrate 24 Gm./sq. m.	1660	1.388	92.5	0.34	83.0
4	Chilean nitrate 48 Gm./sq. m.	1900	1.562	92.0	0.352	82.5
5	Chilean nitrate 60 Gm./sq. m.	1900	1.590	92.0	0.355	82.0
6	Control	1390	1.220	93.5	0.320	84

third to one-half that of the sunshine-grown plant. (*f*) The moisture and alkaloidal content of the shade-grown plants increase on exposing them to the sun. The mean values of the results obtained from several experiments are given in Table III.

TABLE III.—SHOWING THE INCREASE IN THE MOISTURE AND THE ALKALOIDAL CONTENT OF THE SHADE PLANTS ON EXPOSURE TO THE SUN

Period of Exposure to the Sun	Shade Plantlets		Exposed Plantlets	
	Alkaloids, %	Moisture, %	Alkaloids, %	Moisture, %
One week	0.57	89.0	1.13	94.2
Two weeks	0.66	88.4	1.36	91.5

Season.—The plant was cultivated by sowing the seeds in plots of a sandy-loam soil at the beginning of each month for a year. The irrigation of the plots was regulated according to the prevailing atmospheric conditions. It was invariably stopped after the third month of the growth of the plant.

The green flowering plants were collected, and their alkaloidal and moisture contents determined.

The mean values of the results obtained are given in Table IV (*a*) and (*b*). From Table IV one may conclude that:

(*a*) The summer season is the most suitable, spring and autumn are favorable, while winter is entirely unsuited for the cultivation of the plant.

(*b*) Plants sprouting in spring and summer attain flowering after about three months, give average vegetative yields and contain high alkaloidal percentages; while plants sprouting late in summer and in autumn give greater vegetative yield and contain lower alkaloidal percentages.

(*c*) August and September plants give the highest vegetative yield and the lowest alkaloidal percentages. It is noticed that the roots of these plants contain exceptionally low alkaloidal percentages.

Disease.—It was noticed that plants grown in a soil brought from Kerdassa, near Giza, Egypt, showed weak vegetative growth and low alkaloidal content. These plants were found to possess abnormally swollen, beaded roots (Fig. 1). On examining these roots they were found to be infested by the root-knot nematode (*Heterodera marioni*) (4).

The infested root was found to have an intact

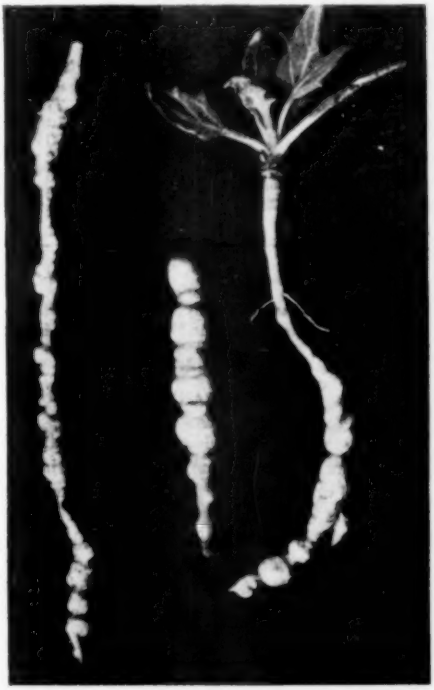

Fig. 1.—Photograph showing the extensive knotting of the root of the plant due to the infestation by the root-knot nematode. The plant at the right is three months old; others lucky enough to escape invasion attain flowering within this time. Natural size.

TABLE IV (A).—SHOWING THE MONTHS OF CULTIVATION AND OF FLOWERING, THE VEGETATIVE YIELD IN GM./SQ. M. OF THE AIR-DRIED MATERIAL, AND THE ALKALOIDAL CONTENT CALCULATED ON MATERIAL DRIED AT 100°

Month of Cultivation	Month of Flowering	Approx. Yield in Gm./Sq. M. of Air-Dried Material	Overground Part of Plant		Underground Part of Plant	
			Total Alkaloids, %	Moisture, %	Total Alkaloids, %	Moisture, %
May	August	1428	1.20	94.0	0.350	84.0
June	September	1428	1.30	94.0	0.350	84.0
July	October	1428	1.20	94.0	0.330	84.5
August	February	2380	0.86	93.0	0.075	80.0
September	Early in March	2143	0.80	93.5	0.090	80.0
October	March	1547	1.00	94.5	0.250	84.5
November	Later in March	1547	1.00	94.2	0.270	84.5
December	...	..	..	..	...	..
January	...	..	..	..	...	..
February	...	..	..	..	...	..
March	July	1428	1.00	94.2	0.300	84.0
April	July	1428	1.10	95.2	0.300	84.0

NOTE: The mean monthly data of the meteorological conditions of cultivation during the experimental period are indicated in Table IV (B).

TABLE IV (B).—THE MEAN MONTHLY DATA OF THE METEOROLOGICAL CONDITIONS OF CULTIVATION DURING THE EXPERIMENTAL PERIOD

Month of Cultivation	Mean of Temperature, ° C.		Mean of Relative Humidity		Mean of Pressure, Mm. of Hg	Duration of Bright Sunshine		Earth Temperature at Depth of 60 Cm., ° C.
	Max.	Min.	8 Hr.	20 Hr.		% Possible	Mean in Hours	
May	34.1	15.1	65	41	737.38	87	11.8	24.9
June	36.7	19.0	69	40	755.68	86	12.1	27.7
July	36.1	20.3	74	47	754.64	86	12.0	29.1
August	34.4	19.3	67	56	755.44	89	11.7	29.4
September	32.7	16.7	71	63	757.74	85	10.5	28.1
October	31.5	15.6	72	66	759.36	77	8.8	26.3
November	26.4	11.7	80	74	760.43	71	7.6	23.0
December	22.0	6.5	92	77	761.00	68	6.9	21.0
January	20.5	5.8	76	70	671.54	64	6.7	17.7
February	21.0	5.1	80	69	762.46	71	7.9	16.8
March	22.4	7.5	72	63	759.66	68	8.1	17.4
April	26.1	9.2	71	58	760.33	77	9.9	19.2

central cylinder, while the hypertrophies characteristic of this disease were found to be localized in the cortical region (Fig. 2).

SUMMARY

The best vegetative yield and highest alkaloidal content are obtained from plants grown in the loamy soil under moderate irrigation. Excessive irrigation is detrimental to the life of the plant and to its alkaloidal content, but the plant survives drought after germination for a long period of time. Plants grown in comparatively dry soil yield a higher alkaloidal percentage than those grown in damp places.

The application of nitrogenous fertilizers, especially the inorganic type, increases the alkaloidal content and the vegetative yield of the cultivated plant.

Sunshine is a fundamental factor for a high normal alkaloidal content of *Hyoscyamus muticus* L. It seems that exposure of shade-grown

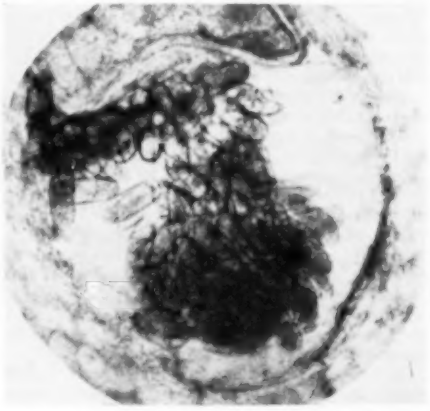

Fig. 2.—A photomicrograph of a transverse section of the infested root passing through the knot, showing the female nematode with great numbers of eggs inside a round cavity in the damaged cortical region. × 300.

plants to the sun is associated with an increase in the metabolic processes within the plant as shown by the rapid formation of hairs, calcium oxalate, coloring matter, and alkaloid.

Spring and summer seasons are the most favorable for sowing the seeds of the plants which flower after three months. Plants sprouting in autumn produce smaller vegetative yield and contain lower alkaloidal percentages than summer plants. Winter is unsuited to the growth of the plant.

Hyoscyamus muticus is liable to infestation by the root-knot nematode (*Heterodera marioni*). The infested plants give very weak vegetative yield and at the same time low alkaloidal percentages as compared with those of the healthy plants.

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Colorimetric and Fluorometric Studies on the Borntraeger Reaction for Anthraquinone Drugs*

By BERNARD V. CHRISTENSEN† and ISMAIL A. ABDEL-LATIF‡

The performance of the Borntraeger reaction on the emodin-containing drugs was reinvestigated. Colorimetric, spectrophotometric, and fluorometric analyses were used as measures to determine the best procedure, reagents, and organi solvents to be employed. The color of the fluorescence under ultraviolet is specific for each of the drugs containing emodin and can be used in their identity. The fluorescence seems to be due to the anthraquinone derivatives and can be used to trace them during extraction procedures.

AMONG the important published modifications of the Borntraeger reaction are those by Cooper and Denston (1), Wallis (2), Fairbairn (3, 4), Tschirch (5), Gilg, Brandt, and Shurhoff (6). These modifications make the test applicable to drugs containing the anthraquinone derivatives, or improve its sensitivity.

EXPERIMENTAL

Tschirch (5) and Gilg, *et al.*, (6), have recommended the use of alkali rather than acid to bring about hydrolysis. The former used an aqueous solution while the latter recommended the use of alcoholic potassium hydroxide solution.

Experimentally, it was found that the alkali hydrolysis is preferable since it gives greater sensitivity to Borntraeger's reaction. Moreover, the anthraquinones are precipitated by the acid and may be partially removed on filtration. They remain in solution in the alkali and thus make it possible to take proper aliquot portions even after standing. Aqueous solution of alkali may cause slight precipitation of the anthraquinones, hence the alcoholic solution of potassium hydroxide is the best hydrolyzing agent.

Under filtered ultraviolet rays, the alcoholic potassium hydroxide extract of Alexandria senna leaves (*Cassia acutifolia* Delile) gave a strong pink fluorescence. On acidification, the anthraquinone derivatives were precipitated and no fluorescence could be observed. On shaking the acidified aqueous extract with ether, the precipitated anthraquinones dissolved in the ether. The ethereal layer showed the strong pink fluorescence while the aqueous layer remained devoid of any fluorescence, even if it was made alkaline. The ether layer was separated and then shaken with ammonia T. S. The ammonia layer showed a distinct rose-red color. The ultraviolet pink fluorescence again moved from the ether layer into the ammonia layer. Consequently, the pink fluorescence under ultraviolet is probably due to anthraquinone derivatives and can serve as an indicator or to trace them during extraction procedures.

If acidic instead of alkaline hydrolysis is used, the filtrate gives no fluorescence at any *p*H. Hence, the alkaline hydrolysis is preferable to the acidic one.

The Choice of the Organic Solvent.—The Borntraeger reaction was performed simultaneously under similar conditions on equal weights of senna leaves, changing only the organic solvent used. Ether, petroleum ether, benzene, amyl alcohol, chloroform, ethylene dichloride, and carbon tetrachloride were compared. The optimum solvent or

* Received April 11, 1949, from the Pharmacognosy Research Laboratory, College of Pharmacy, The Ohio State University, Columbus.
Abstract of dissertation submitted to the Graduate School by I. A. Abdel-Latif in partial fulfillment of the requirements of the degree of Doctor of Philosophy.
Presented to the Scientific Section, A. PH .A., Jacksonville meeting, April, 1949.
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solvents are those which extract all the anthraquinone derivatives from the acidified alcoholic potassium hydroxide extract and, in the meantime, do not retain these anthraquinones when shaken with ammonia, but which yield all of them to the ammonia. Consequently, the organic solvent layer, after being shaken with the acidified extract, should show the highest pink fluorescence, and the aqueous layer (even when separated and made alkaline) should show very slight or no fluorescence. When shaking the separated organic solvent layer with ammonia, the latter should show the highest fluorescence, while the organic solvent layer should show very weak fluorescence, or none.

On this basis and according to the results, the organic solvents are arranged in descending order as follows: Ether (the best); chloroform, ethylene dichloride, amyl alcohol (average); benzene, petroleum ether, and carbon tetrachloride (poor).

Ether and amyl alcohol are the best solvents if stability of color and fluorescence is taken into consideration. However, amyl alcohol freely dissolves aqueous solutions. Thus ether surpasses all other organic solvents tested and is recommended to be used in performing the Borntraeger reaction on senna.

Spectrophotometric Analysis.—Spectrophotometric analysis of the colors obtained in the ammonia layers was also carried out so as to exclude the human-eye judgment as well as to get exclusive accurate comparison. Four different organic solvents were comparatively used, namely, ether, chloroform, ethylene dichloride, and amyl alcohol. The tests on equal weights of senna were similarly and simultaneously carried out. The ammonia layers obtained were spectrophotometrically analyzed, using ammonia T. S. previously shaken with pure ether as a blank. A Cenco-Sheard Spectrophotelometer was used, with a slit opening of 0.75 mm. and no filters. Readings of the per cent transmission (% T) were made at the wave lengths ranging from 440 to 700 mμ, for every sample using small test tubes as sample holders. Using special conversion tables, the corresponding extinction (E) values were obtained for the (% T) readings.

From the results obtained, graphs (Fig. 1) were plotted to show the (E) values against the wave lengths.

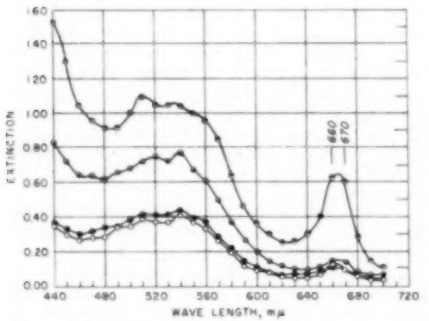


Fig. 1.—Spectrographs of ammonia layers using various organic solvents.

◓ Ether; ◒ amyl alcohol; ● ethylene dichloride; ○ chloroform.

CONCLUSIONS

1. The similarity in the graph pattern and the curves indicate that the organic solvents, though different, extracted the same type of chemical compounds having similar behaviors toward absorption of light.
2. The ether curve is the only one that shows a very distinct peak.
3. All the graphs show a peak around 660 to 670 mμ wave lengths. This band of wave length may be characteristic for the anthraquinone derivatives.
4. The patterns for chloroform and ethylene dichloride are nearly identical.
5. Ether is again indicated as the best solvent.

Investigation of the Flourescent Substance

Quenching.—The fluorescence was found to be quenched by potassium iodide, acids, and water. Potassium iodide is the strongest quenching agent. If a solution of the fluorescent substance is made in a water-miscible solvent, the water-quenching effect is noticed when the water ratio reaches or exceeds one-third that of the total solvent. Acids exerted a stronger quenching effect than water. On the other hand, potassium thiocyanate, M solution, did not quench the fluorescence even with a proportionally great volume.

Chemical Nature of the Fluorescent Substance.—In order to identify the fluorescent substances occurring in the emodin drugs, the behavior of anthraquinone, chrysarobin (crude) alizarin (indicator *p*H 9.5), and Anthralin was investigated. Chlorophyll was also checked to exclude the factor of its presence in the leaves. Phenolphthalein was compared because it gives purple, but not red, color with Borntraeger's reaction and also due to the fact that it is an indicator. From the results obtained, shown in Table I, it is clear that only the anthranols, anthralin and chrysarobin, give fluorescence. This indicates that hydroxyl groups must be present in the structure. In the case of alizarin, which does not show fluorescence, the two hydroxyl groups are in 1, 2 positions, while in the other two compounds, the hydroxyl groups are in 1, 8, 9 positions which exactly resembles the structure of the naturally occurring principles.

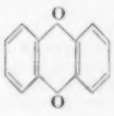
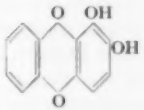
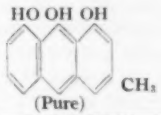
Consequently, the fluorescence is most probably due to the hydroxy anthraquinone derivatives.

Sensitivity of the Color and Fluorescence.—The color of the ammonia layer obtained when Borntraeger's reaction is performed on senna leaves is found to be sensitive to 0.0004 Gm. of the crude drug. If it is assumed that the drug contains as high as 10% of anthraquinone derivatives, then the test is actually sensitive to at least $^1/_{25,000}$ dilution of these compounds.

However, the fluorescence was not equally sensitive. It is sensitive to at least 0.006 Gm. crude drug or to $^1/_{1500}$ dilution of the active constituents. Hence, both color and fluorescence are sensitive tests.

Comparing All the Emodin Drugs.—The other members of the emodin group of drugs were simultaneously checked to determine whether or not they

TABLE I.—EFFECT OF STRUCTURE ON FLUORESCENCE

Compound	Borntraeger's Reaction	Fluorescence	Formula
Chlorophyll	Negative	None (solns., in alcohol, acetone and alc. KOH)	
Phenolphthalein	Negative (Gives violet not red)	None (at any *p*H value)	
Anthraquinone	Negative	None	
Alizarin	Negative (Ammonia layer is purple at *p*H 10, brown at *p*H 6)	None	
Anthralin	Positive (Deep blood red)	Very weak pink	
Chrysarobin (Crude)	Positive (dark blood red)	Light brown	 (Pure)

behave similarly to senna under ultraviolet during the performance of Borntraeger's reaction.

Aloe (cape variety) and aloin gave yellow, cascara gave light pinkish yellow, while rhubarb and frangula gave pink fluorescence like that of senna but not as strong. Crude chrysarobin gave light brown while *rumex* and *Rhamnus carthartica* gave light green fluorescences.

Consequently, the color of the fluorescence varies with the particular drug of the group and thus can be used for identity even in mixtures.

SUMMARY

1. The reinvestigation of the present modifications of the Borntraeger reaction for the anthraquinone drugs has shown that alkali hydrolysis is preferable to acid hydrolysis. Alcoholic potassium hydroxide solution is recommended.

2. Ether was found to be the best organic solvent, especially in the case of senna leaves.

3. The fluorescence under ultraviolet, like the anthraquinone derivatives, moved from the aqueous solution, organic solvent layer, to the ammonia layer during the application of the test.

4. Hydroxyl groups must be present in 1,8-positions of the anthraquinone structure in order that the derivative give a positive Borntraeger reaction as well as the fluorescence.

5. The fluorescent substances are most probably the hydroxy anthraquinone derivatives. Hence, fluorescence can be used to trace them as well as to determine the end point during extraction procedures.

6. Identity tests based on the color of the fluorescence given by the emodin drugs with Borntraeger's reaction may be established.

7. The fluorescence may be used to detect mixtures of these drugs based on color of fluorescence.

8. The color of the ammonia layer is stable under certain conditions. It is sensitive to at least 0.0004 Gm. crude drug or to 1 in 25,000 dilution of the active constituents.

9. The fluorescence is sensitive to at least 0.006 Gm. crude drug or to 1 in 1,500 of the active constituents.

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A New Microtechnique in Ultraviolet Analysis*

By BERNARD V. CHRISTENSEN† and ISMAIL A. ABDEL-LATIF‡

A technique of using capillary tubes as sample holders to expose solutions under filtered ultraviolet light has been devised and the procedure used is described.

IT WAS OBSERVED that the fluorescence was more distinct on the surface or meniscus of the solutions than in its depth or body when examined under ultraviolet rays. As a general rule, it was obvious that the wider the container used, the clearer and more distinct the fluorescence. Hence, the depth of the solution has no advantage or particular value; but, on the other hand, it may give a wrong conception and lead to misinterpretations due to the interference of the color of the solution.

As a result of these observations, the idea occurred to use "Capillary Tubes" filled with the solutions. It is suggested here that this new technique be designated: "The Capillary Tube Method for Ultraviolet Analysis."[1]

PROCEDURE

Preparation of Capillary Tubes.—Ordinary soft glass tubing can be used if it does not by itself show any appreciable fluorescence when exposed to ultraviolet light. Glass tubing of about 6 mm. outside diameter and about 4 mm. inside bore diameter is cut into pieces, each about 8 inches long. Each piece is fire polished at both ends and then pulled out into capillary tubes as that shown in Fig. 1. By the aid of a microcaliper, those capillaries that vary from the required lumen or bore width can be rejected. The wide tubing end is quite useful in pipetting the sample. It also is convenient for purposes of labeling and holding. However, it may be advisable to cut off this wide end if the capillary needs to be sealed for permanent or long storage and preservation of samples for future reference.

Advantages of the Capillary Tube Method.—This new microtechnique has several great advantages in the ultraviolet analysis. Some of the important advantages are listed below:

(*a*) The use of the capillary tube when filled with solution allows a view of a great length of the surface of the solution. This is equal to a large surface area or width of meniscus in other containers.

(*b*) There is practically no depth to the solution which would otherwise interfere wth the interpretation of the color of fluorescence and lead to wrong conclusions.

(*c*) It allows the comparison of several capillary tubes at one time (Fig. 2.).

(*d*) It simplifies the drawing of samples from the original solution without disturbing the progress of the experiment. Samples can be pipetted out at any stage, as well as from either the top or bottom layer of immiscible solutions.

Fig. 2.—Fluorescence under ultraviolet lamp (the new microtechnique, using the capillary tubes).

Fig. 1.—Capillary tube.

* Received April 4, 1949, from the Pharmacognosy Research Laboratory, College of Pharmacy, The Ohio State University, Columbus.
Abstract of part of dissertation submitted to the Graduate School by I. A. Abdel-Latif in partial fulfillment of the requirements of the degree of Doctor of Philosophy.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

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[1] The new technique should not be confused with the one already known, which is usually called: "The capillary analysis under ultraviolet." This latter nomenclature refers to the fact that the method makes use of the capillarity of filter paper fibers when absorbing solutions. The filter paper is then dried and examined under ultraviolet light.

(*e*) The method makes possible the use of the ultraviolet analysis in the microchemical field. Only two drops of a solution are enough to fill the capillary tube.

(*f*) The method can be used for the quantitative comparison of fluorescence intensity.

(*g*) Solutions or samples are kept from rapid evaporation, thus preventing changes in their concentrations. It also prevents or diminishes rapid changes in the nature of the colors, fluorescence, and chemical composition due to open contact with the air.

(*h*) Storing, preservation, and stabilization of samples are rendered possible using this technique. The capillary tubes can be fire sealed even when they contain ethereal or other inflammable solutions. Samples of ethereal solutions of anthraquinone derivatives have been kept unchanged for six months in such sealed capillaries.

SUMMARY

A new microtechnique for the ultraviolet analyses has been devised and described. Accurate and rapid qualitative and quantitative results are obtainable on a microscale by the use of this technique.

Studies on the Identification of Narcotics. I. Microchemical Reactions for Identification of Metopon*

By C. G. FARMILO† and G. H. W. LUCAS‡

Qualitative microchemical reactions for the identification of Metopon (methyldihydromorphinone) have been studied, based on the functional groups of the molecule. It has been found that Metopon will give a positive Janovsky reaction. The ketone group can be reduced to an alcoholic hydroxyl with zinc and hydrochloric acid; the resulting methyldihydromorphine will give a positive Sanchez reaction. A specific reaction was found using sodium nitroferricyanide and sodium hydroxide; Metopon gives a red color whereas Dilaudid, Dicodid, Eucodal, and Methadone do not. A modification of the Marquis reagent gives specific color developments which can be used to distinguish the ketones. Several coupling and dye formation reactions gave distinctive colors. The triphenylmethane-type dyes were obtained when the phenol and phenylether narcotics were condensed in the presence of concentrated sulfuric acid with benzaldehyde derivatives.

METOPON is one of the new analgesics that has become available. It was first synthesized by Small, Fitch, and Smith (1) in the course of a study of the reactions of thebaine with organo-magnesium halides. The methyldihydromorphinone molecule is a new type of morphine derivative. Pharmacologically in comparison with dihydromorphinone, its nearest relative chemically speaking, Metopon exhibits an exaggeration of analgesic effectiveness and diminution of sedative, euphoric, emetic, and intestinal actions. The National Research Council of the United States, about two years ago, arranged for the distribution of Metopon under its supervision in a large clinical experiment in which the drug was made available to one class of patients, cases of terminal malignancy. It is not clearly established from these experimental results (2) whether patients who have never received any narcotics will develop an addiction to Metopon, but it seems possible that the drug is slower in producing dependence than other morphine derivatives, and also incompletely supports an established morphine dependence. A thorough review of the clinical evidence and the pharmacology of Metopon is given by Eddy and Houde and others (2). From the point of view of the Canadian Food and Drug Administration, Metopon, being a derivative of morphine, is a narcotic, and tests were needed for its identification; thus the present work was undertaken.

Metopon chemically is related to other ketone derivatives of morphine and codeine, namely Dilaudid and Dicodid. Another ketone derivative of codeine, made by German chemists, is dihydrohydroxycodeinone, which they named Eucodal. It is most closely related to Dicodid, differing from the latter by the introduction of a hydroxyl group at C-14. All of these compounds

* Received February 16, 1949, from the Department of National Health and Welfare, Ontario, Canada.

The authors wish to acknowledge with thanks the many suggestions and assistance of Dr. L. I. Pugsley, Chief of Laboratory Services, Food and Drug Division, Ottawa, Ontario.

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have in common the fact that they are dihydro derivatives of morphine and codeine and that they have a ketone group at C-6. Of the recent analgesics the Methadones and Isomethadones are also ketones and must be considered in chemical reactions for purposes of identification, since they may give similar reactions to Metopon.

The structural feature which distinguishes Metopon, chemically, from the other keto morphine and codeine derivatives such as Dilaudid, Dicodid, and Eucodal, is the C-7 methyl group, that is a methyl adjacent to the carbonyl radical. Here, as in the case of alpha-methyl substituted aldehydes, acetaldehyde, and crotonaldehyde, we have the possibility of hyperconjugation (3) of the methyl group with the carbonyl. This structural feature produces changes that have been thoroughly established (4), in both physical and chemical reactivity. For the present, chemical reactions of Metopon determined by the following structural groups will be discussed: (*a*) cyclic ketone, (*b*) methyl group, alpha to the ketone, and (*c*) phenol hydroxyl. In these reactions Metopon is compared with Dilaudid, Dicodid, and Eucodal and racemic Methadone and at the same time the similar reactions of morphine and codeine are reviewed.

EXPERIMENTAL

Materials.—The Metopon hydrochloride used in these experiments was supplied by Parke, Davis and Co., Walkerville, Ontario. Methadone was the racemic compound supplied by Burroughs Wellcome & Co., Montreal, P. Q., under the trade name Physeptone. Dilaudid hydrochloride and Dicodid bitartrate, products of the I. G. Farbenindustrie, Ludwigshafen, were obtained from Knoll Limited, London, England. All of these compounds were used without further purification.

Identification Reactions of Metopon

1. Detection of the Keto Group.—The Janovsky reaction, consisting of the condensation of *m*-dinitrobenzene with a ketone in the presence of sodium hydroxide, produces colors varying from a lilac to a raspberry-red. The reaction was first used with acetone and later by Péronnet and Truhaut (5) to identify other aldehydes and ketones. Pesez (6) has modified the reaction for the detection of the benzene nucleus in alkaloids. Ekkert (7) used the reaction to identify Eucodal. Denoel and Soulet (8) have applied the reaction to the identification of Dilaudid and Dicodid. The following modification of the Janovsky reaction has been adopted to indicate the keto group in Metopon:

Method.—One drop of an alcoholic solution of *m*-dinitrobenzene (10:1) was added to the alkaloid (0.1 mg. of the solid) in question. The solution was aided by stirring with a glass rod, and then a drop of aqueous sodium hydroxide (20%) was added. The color developed was characteristic of the particular ketone used. In Table I the colors obtained with acetone, Metopon, Dilaudid, Dicodid, Eucodal, and Methadone are compared.

TABLE I.—COLORS OBTAINED IN THE JANOVSKY REACTION WITH ACETONE, METOPON, DILAUDID, DICODID, EUCODAL, AND METHADONE

Ketone	Color Change with *m*-Dinitrobenzene plus Sodium Hydroxide Solution		
Acetone	Blue purple	Red purple	Brown
Metopon	Mauve	Dark purple	Blue
Dilaudid	Light purple	Yellow	Tan
Dicodid	Red purple	Yellow	Black
Eucodal	Light red purple	Tan	Brown
Methadone	Delayed purple	Medium purple	Purple

Further proof of the presence of a keto group in any of these narcotics except Methadone can be obtained by the use of the Sanchez reaction following reduction of the keto group to an alcoholic hydroxyl. The keto group of Methadone requires special conditions for its reduction (9) and the following test is not applicable to this analgesic as it is described. The Sanchez reagent is prepared by dissolving vanillin (0.3 mg.) in concentrated hydrochloric acid (100 ml.)

Reduction Method.—A small amount (0.1 mg.) of the solid alkaloid was dissolved in concentrated hydrochloric acid (5 drops) and water (2 drops) was added. The micro-crucible was placed on a water bath and to it granulated zinc (0.25 Gm.) was added and the mixture heated for a few minutes or until the evolution of hydrogen had ceased. Additional water was added to maintain the volume of liquid at its original level in the crucible. The liquid was then decanted into another micro-crucible and the Sanchez reagent was added (2 drops) and the solution then warmed until the characteristic color developed. The limit of sensitivity of this reaction was 0.1 mg. of the solid alkaloid. The colors obtained faded very rapidly. The colors are shown for morphine, codeine, Metopon and Dilaudid, Dicodid, and Eucodal in Table II.

Neither of the chemical reactions reviewed so far do more than generally classify Metopon as a ketone. As has previously been stated the presence of the methyl group on the C-7 modifies the reactivity of the carbonyl group as it exists in the alicyclic ketones of nonalkylated morphinones. Sodium nitroferricyanide reacts with methylketones, such as acetone,

TABLE II.—COLORS OBTAINED WITH MORPHINE, CODEINE AND AFTER REDUCTION WITH METOPON, DICODID, DILAUDID, AND EUCODAL AND THE SANCHEZ REAGENT

Narcotic	Color with Vanillin Plus Hydrochloric Acid
Morphine	Light purple
Codeine	Purple
Metopon	Colorless
Dilaudid	Colorless
Methyldihydromorphine	Violet
Dihydromorphinone	Mauve
Dicodid	Colorless
Eucodal	Colorless
Dihydrocodeinone	Purple
Hydroxydihydrocodeinone	Light purple

in the presence of alkali (10) to produce red colors. Ketones in which the carbonyl is on the C-3 carbon atom such as Methadone do not give colors with this reagent. Thus Metopon is the only one of the ketone morphine derivatives which gives a color with this reagent, and the color can be obtained with one gamma of the solid.

Method.—After extraction and evaporation of the solvent in a white porcelain crucible, one drop of a solution of a freshly prepared sodium nitroprusside (5%) in water was added. The reagent was mixed thoroughly with the alkaloid and a drop of aqueous sodium hydroxide. After a few seconds in contact with the reagents, Metopon produced a red color which faded in about five minutes to a bright yellow-orange color. This test can be recommended as a specific test for the alkaloid Metopon, as it is distinctive for this ketone, and distinguishes it from other morphinones and codeinones, as well as from the new synthetic Methadone.

2. Detection of the Phenolic Hydroxyl.—Metopon, as well as being readily soluble in alkali and in solutions of sodium salts, gives the classic phenol reaction with ferric chloride. Thus morphine, Dilaudid, Eucodal, and Metopon give varying shades of blue with ferric chloride. It is to be noted that the color of the precipitate is changed by the acid with which the quaternary salt is formed: for example, morphine-free base gave a dark blue green; morphine hydrochloride, light green solid not completely soluble; morphine sulfate, light green-blue, completely soluble; morphine bitartrate, light green. A modification of the hydroxyl reactivity in the morphine phenols is shown by the fact that ferric sulfate, which ordinarily, i.e., with hydroxy benzenes, does not give a blue color, does so in the case of the narcotics.

Method.—The solid alkaloid (0.1 mg.) was placed in a micro-crucible after extraction and evaporation of the solvent. The final step in the evaporation was carried out in the crucible. One drop of the ferric salt, chloride, or sulfate reagent (5%) aqueous solution, was added and the mixture stirred until the color had developed. The colors developed varied with the individual alkaloid. Metopon gave an ultramarine blue with ferric chloride.

Fulton (11) developed a reaction for the identification of small amounts of formaldehyde using morphine combined with an oxidizing agent, such as ferric sulfate and sulfuric acid. A modification of this reaction has been found useful in determining Metopon. The first step is to mix the alkaloid with a drop of ferric sulfate solution. One drop of a freshly prepared Marquis reagent is added carefully to the exact center of the ferric sulfate-alkaloid solution or mixture. An immediate color developed which in time gave rise to striations which were colored, and the color varied for each of the alkaloids.

It is well known that phenols couple with diazotized, aromatic amines to yield dyes. Many modifications of this reaction have been suggested, using a variety of amines as coupling agents. Lautenschlager (12) and David (13) have used the diazotized sulfanilic acid as the coupling reagent. Diazotized aniline has been used by Fiegl (14) in the identification of phenols, and works equally well with morphine-like molecules. The method of David has been modified, using sulfanilamide as the coupling reagent.

Method.—Dissolve sulfanilamide (0.01 Gm.) in 1 cc. of water and add a solution of sodium nitrite (4 drops of 1% aqueous solution) and hydrochloric acid (5 drops). When the solution is complete and one has obtained a diazotized solution of sulfanilamide, add one drop to the alkaloid either as the solid or in solution. Metopon gives a carmine-red on the addition of one drop of aqueous sodium hydroxide (20%) to the above solution containing the Metopon. The other ketones give distinctive colors varying from red to bright orange.

Liebermann (15) has shown that a large number of phenols form nitroso derivatives with nitrous acid. Kramer (16) and Brunner and Chuit (17) have shown that these nitroso derivatives, with an excess of the phenol, form dyestuffs by the elimination of a molecule of water. Wieland and Kappelmeier (18) have applied this reaction to morphine. Radulescu (19) has applied the same reaction using a concentrated hydrochloric acid solution of nitrous acid instead of sulfuric acid in the identification of morphine. Denoel and Soulet (20) have applied a modification to the identification of Dilaudid. The reaction as it has been used with Metopon is as follows:

Method.—A drop of the etheral test solution was allowed to evaporate to dryness in a micro-crucible, treated with sodium nitrite (0.1 mg.), and concentrated hydrochloric acid (1 drop) was added. Characteristic colors with various alkaloids are produced at this stage. Codeine does not give a color. While fumes of nitric oxide were still being evolved, one drop of sodium hydroxide solution was added and immediate formation of red-colored dyes was brought about. On standing, these colors changed to purples and oranges, characteristic of the particular alkaloid.

Phenols and phenyl ethers condense with aromatic aldehydes to form triphenylmethane derivatives, Baeyer (21). The products formed are the leuco-compounds of well-known dyes. When pure, these leuco-compounds dissolve in acids or alkalies to give colorless solutions. However, in an oxidizing medium the dye is formed by oxidation of the base. This occurs in acid solution, two hydrogens being removed from the *ortho* position in the case of phenols with blocked *para* positions. All triphenyl methane dyes dissolve in concentrated sulfuric acid with an orange color exactly like triphenylcarbinol itself as shown (22). By diluting this solution with water a colorless solution for the triacid benzenoid salt can be obtained. The color change produced by acids can be reversed by careful addition of alkali. On the addition of a few drops of sodium hydroxide solution to the aqueous solution of a basic triphenylene dye, a faintly colored flocculent precipitate separates. This is the carbinol to which repeated reference has already been made which, when pure, is colorless. When the colorless carbinol base is treated with acid, the quinoid structure is reformed and the dye is produced. On dissolving cautiously with cooling, it can be seen that the color attains its maximum depth only gradually. Hence the exceedingly unstable colorless carbinol salt is first formed, which changes into the dye with spontaneous elimination of water.

A series of 16 different aromatic aldehydes have been condensed with Metopon, morphine, codeine, and heroin, in the presence of concentrated sulfuric

acid to produce triphenylmethane-type dyes in the majority of cases. It is interesting to note also that the presence of auxochromes on the aromatic nucleus of the aldehyde modifies the color produced in the reaction. For example, benzaldehyde with four narcotics produces purples of various shades, while *p*-dimethylaminobenzaldehyde yields brilliant red-orange colors. The colors are shown in Tables III and IV.

further study of the quantitative aspects of the color-forming reactions. The experimental results for this will be reported later.

SUMMARY AND CONCLUSIONS

Metopon has been shown to undergo characteristic microchemical reactions which are useful for the purpose of qualitative identification.

TABLE III.—LIEBERMANN'S NITROSO REACTION WITH METOPON, MORPHINE, CODEINE, AND HEROIN SHOWING ACID-BASE REACTIONS

	Reagent Used	Metopon	Morphine	Codeine	Heroin
1.	$NaNO_2$ + HCl (15%) + Narcotic	Yellow	Red-purple	Colorless	Light purple
2.	$NaNO_2$ + HCl + Narcotic + NaOH (40%)	Red-orange	Brown	Colorless	Light purple
3.	$NaNO_2$ + HCl + Narcotic + NaOH + H_2SO_4 (98%)	Yellow	Yellow	Yellow	Light purple
4.	$NaNO_2$ + HCl + Narcotic + NaOH + H_2SO_4 + NaOH	Red on yellow	Colorless	Yellow	Faint purple

TABLE IV.—TRIPHENYLMETHANE DYE-TYPE REACTION OF NARCOTICS

Metopon	Morphine	Codeine	Heroin	Reagent + Sulfuric Acid
Faint yellow	Med. yellow	Bright yellow	Yellow	*o*-Nitrobenzaldehyde
Faint yellow	Yellow-orange	Orange	Purple	*m*-Nitrobenzaldehyde
Orange on purple	Light purple	Red-purple	Blue-purple	*p*-Chlorobenzaldehyde
Yellow	Yellow	Orange	Orange-brown	*m*-Hydroxybenzaldehyde
Colorless	Faint purple	Speck purple	Deep purple	*p*-Hydroxybenzaldehyde
Orange	Red-orange	Orange-purple speck	Yellow with purple	*o*-Hydroxybenzaldehyde
Light purple	Purple	Bright purple flash	Purple fading	*p*-Methoxybenzaldehyde
Colorless	Colorless	Colorless	Purple (flashed)	3-Methoxy-4-hydroxybenzaldehyde
Red purple	Red-purple	Red-purple	Faint red purple	*o*-Chlorobenzaldehyde
Blue-purple light	Blue-purple med.	Blue-purple dark	Blue-purple dark	5-Nitro-3-methoxy-4-hydroxybenzaldehyde
Colorless	Colorless	Colorless	Purple	3,4,5-Trinitrobenzaldehyde
Orange	Dark orange	Orange	Light orange	*p*-Dimethylaminobenzaldehyde
Dark purple	Dark purple	Dark purple	Dark purple	Formaldehyde
Purple-black	Purple lighter	Purple-brown	Purple	Acetaldehyde
Yellow	Grayed yellow	Grayed purple	Dark red Purple	Benzaldehyde
Orange	Yellow-orange	Red-purple	Blue-purple	Cinnamaldehyde

DISCUSSION

On the basis of the chemical reactions given above it is possible to separate Metopon, by its reactive ketone group, from morphine and derivatives containing alcoholic hydroxyl as well as keto groups. Also by means of the specific sodium nitroferricyanide reaction in sodium hydroxide, which identifies Metopon as an alpha-methyl substituted ketone, the alkaloid can be distinguished from other morphinones such as Dilaudid, Dicodid, and Eucodal and also from the synthetic analgesic Methadone.

The evidence from the formation of a triphenylmethane-type leuco-base indicates that Metopon undergoes a well-known type of dye reaction when condensed with an aromatic aldehyde or an aliphatic aldehyde in the presence of concentrated sulfuric acid. Further indications suggest a similar mechanism for the well-known Marquis reaction.

Further work is under way to modify the latter reactions, to use them in spectrophotometric quantitative estimation of Metopon. This involves

1. The Janovsky reaction with *m*-dinitrobenzene is used to identify the ketone group.

2. The Sanchez reaction distinguishes Metopon categorically from morphine and codeine, since reduction is required before Metopon will react with vanillin in hydrochloric acid.

3. The reaction of sodium nitroferricyanide in sodium hydroxide is specific for Metopon.

4. The phenolic group of this molecule is characterized by a modified Marquis reaction using an added mild oxidizing agent. The specific color developments in this reaction, with time aid in characterizing all the morphinones.

5. Coupling reactions with diazotized sulfanilamide and the molecule Metopon, dependent on the phenolic hydroxyl, are described.

6. A dye-forming reaction with nitrous acid has been shown to give characteristic colors with Metopon.

7. A new series of dye-forming color reactions have been developed, dependent upon the formation of triphenylmethane-type compounds. Metopon will undergo such a reaction when it is condensed with aromatic aldehydes, benzaldehyde derivatives in concentrated sulfuric acid.

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Refractive Index Measurements At and Above the Melting Point of Solids, Gums, Resins, and Waxes*

By J. DOBRAN†, M. M. ACKER†, and H. A. FREDIANI‡

Melting points and refractive indices at the melting points have been determined for a large number of gums, waxes, and resins. These criteria of purity are reported in this paper and are believed to be applicable for rapid identification purposes.

THE SIMULTANEOUS determination of refractive index and melting point has been suggested (1) as a rapid and convenient method for the identification of substances with melting points below 200°. The method has proved reliable in a laboratory frequently called upon to certify the nature of various natural products. Since these data have been of use to various other laboratories, it is believed that their publication would be justified at this time. The natural products studied are of primary interest to pharmaceutical and clinical laboratories.

EXPERIMENTAL

The instrument used for these determinations is the Fisher Refractometer (2) supplied with heating head (Fig. 1). This instrument converts the sample to be measured into a "liquid prism." Simultaneous observation of an illuminated slit and its virtual image, as seen through the "liquid prism," permits measurement of the vertical displacement of the image from the slit. This displacement is a measure of the refractive index of the sample.

In actual practice, a small crystal or particle of solid is placed in intimate contact with the glass prism supplied with the instrument. The temperature is gradually raised. Because of the relative opacity of solid materials, no slit image is visible until the melting point of the sample is reached. At this point, the solid melts, a liquid prism is formed, and a clear-cut image appears. For waxes, this phenomenon permits a reproducible determination of the melting point, accurate to 1–2°. Refractive indices are determinable to ±0.002 unit. Complete details of the technique involved have been discussed elsewhere (1).

Fig. 1.—Fisher refractometer, supplied with heating head.

* Received March 20, 1949, from Merck & Co., Inc. Rahway, N. J.
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RESULTS

The simultaneous determination of two physical constants does much to characterize clearly a sample. The data included in Table I have been of much use in identifying a complex natural substance with a minimum of expenditure of time and effort. Most of the refractive index measurements listed herein have not been previously reported in the literature. The melting points obtained by this method are more clear cut and reproducible than those obtained by the techniques generally employed (ring and ball method) for waxes and similar products that are not crystalline.

In each case, samples from at least two different lots were studied. However, the difficulty experienced in attempting to trace the history of these natural products was so great that upper and lower possible limits could not be set. For example, four different samples of Candelilla Wax were measured and the melting point of each found to be 66°. There was no way of tracing to see whether these four samples represented a single original lot of material or not. Since the purchase dates of these

TABLE I.

Compound	Melting Point, °C. Detd.	Handbook[a]	Refractive Index of Melting Point Detd.	Handbook	Dispersion Measurements Red	Green	Scale No.
Aloe Cape	155	.....	1.606	.....	1.598	1.613	2
Aloe Curacao U. S. P.	155	.....	1.600	.....	1.595	1.607	2
Aloe Socotrine U. S. P.	210	215	1.518	.....	White image		
Amber (Clear)	Above 300	.....	No data recorded		...	...	.
Amber (Crude)	Above 300	.....	No data recorded		...	...	.
Asphaltum (Calif.)	160	.....	No data recorded		...	...	.
Asphaltum (Egypt)	150	.....	No data recorded		...	...	.
Bayberry Wax (Myrtle)	50	47–48	1.450	1.4511 @40°	White image		.
Beeswax (Yellow) (U. S. P.)	63	62–65[b]	1.450	1.455–1.459 @40°	White image		.
Beeswax (White) (U. S. P.)	63	62–65[b]	1.450	1.455–1.459 @ 40°	White image		.
Beeswax (White) (T. L.)	63	.....	1.450	1.455–1.459 @ 40°	White image		.
Candelilla Wax	66	68–70[b]	1.474	.....	White image		.
Carnauba Wax	84	84–86[b]	1.455	1.467–470 @ 40°	White image		.
Ceresin (White)	73	.....	1.436	.....	White image		.
Ceresin (Yellow)	55	74–80[b]	1.440	.....	White image		.
Ceresin (Black)	83	61–78[b]	1.452	.....	White image		.
Cocoa Butter	37	48–53	1.462	1.454–1.458 @ 40°	White image		.
Gum Ammoniac	85–90	.....	1.549	.....	1.545	1.554	1
Gum Anime	Above 300	.....	No data recorded		...	...	.
Gum Arabic (Amber Sorts)	Above 300	.....	No data recorded		...	...	.
Gum Arabic (Select 1)	Above 300	.....	No data recorded		...	...	.
Gum Arabic (Select 2)	Above 300	.....	No data recorded		...	...	.
Gum Arabic (Select 3)	Above 300	.....	No data recorded		...	...	.
Gum Asafœtida	65	.....	1.560	.....	1.555	1.565	1
Gum Benzoin	85	< 100	1.594	.....	1.585	1.600	2
Gum Camphor (Domestic)	47	.....	1.482	.....	White image		.
Gum Camphor (Japan)	33	174–177[b]	1.486→	.....	White image		.
Gum Catechu	Above 300	.....	No data recorded				
Gum Cherry	Above 300	.....	No data recorded				
Gum Copal (Zanzibar)	195	300	1.516	.....	White image		.
Gum Damar (Batavia)	87 (East Indian Variety)	.....	1.523	.....	White image		.
Gum Damar (Singapore)	125	100	1.515	.....	White image		.
Gum Elemi	85–95	Soft	1.516	.....	White image		.
Gum Euphorbium	85	.....	1.524	.....	White image		.
Gum Gamboge	150–155	1.548	...	.....	1.540	1.563	3
Gum Gambir	Above 300	.....	No data recorded				
Gum Ghatti (Select 2)	Above 300	.....	No data recorded				
Gum Guaiac	106	85–90	1.598	.....	1.594	1.605	2
Gum Indian	Above 300	.....	No data recorded		...	...	.
Gum Locust Bean	Above 300	.....	No data recorded		...	...	.
Gum Mastic (N. F.)	80–85	95	1.517	.....	White image		.
Gum Myrrh	Above 300	.....	No data recorded		...	...	.
Gum Olibanum	Above 300	.....	No data recorded		...	...	.

(*Continued on p. 497*)

TABLE I. *(Continued from p. 496)*

Compound	Melting Point, °C. Detd.	Handbook[a]	Refractive Index of Melting Point Detd.	Handbook	Dispersion Measurements Red	Green	Scale No.
Gum Seed Lac.	90	.....	1.505	.....	White image		.
Gum Spruce	90	.....	1.580	.....	1.565	1.590	4
Gum Tragacanth	Above 300	.....	No data recorded				
Japan Wax	48	53.0–56.5	1.456	1.456–1.459 @ 40°[b]	White image		.
Lard (Benzoinated)	47	.....	1.462	.....	White image		.
Lard (U. S. P.)	28	36–42[b]	1.466	1.461–1.462	White image		.
Lecithan (From Eggs)	97	.....	1.459	@ 40°	White image		.
Lecithan (From Veg.)	30	.....	1.481	.....	White image		.
Montan Wax (Refined)	83	80–86	1.438	.....	White image		.
Paraffin, Imported (40–45)	42	42–60[c]	1.440	.....	White image		.
Paraffin (55–60)	55	.....	1.440	.....	White image		.
Paraffin (60–62)	57	.....	1.439	.....	White image		.
Paraffin (68–72)	67	.....	1.442	.....	White image		.
Paraffin N. F., soft	48	.....	1.440	.....	White image		.
Paraffin N. F., med.	50	.....	1.440	.....	White image		.
Paraffin N. F., hard	56	.....	1.439	.....	White image		.
Paraffin N. F., hard (60–62)	61	.....	1.439	.....	White image		.
Rosin (Dark)	70	120	1.532	.....	1.530–1.535		1
Rosin (Light)	65	135	1.531	.....	1.529–1.533		1
Scammony	130	.....	1.469	.....	White image		.
Shellac (Orange)	85–90	150	1.501	.....	White image		.
Shellac (White)	Above 300	.....	No data recorded				
Tallow (Mutton U. S. P.)	49	33.5–49	1.456	1.455–1.459 @ 40°	White image		.
Tissuemat (50–52)	51	.....	1.441	.....	White image		.
Tissuemat (52–54)	53	.....	1.441	.....	White image		.
Tissuemat (54–56)	53	.....	1.440	.....	White image		.
Tissuemat (56–58)	55	.....	1.440	.....	White image		.
Tissuemat (60–62)	58	.....	1.439	.....	White image		.

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[b] The Merck Index, Merck & Co., Inc., Rahway, N. J., ed. 5, 1940.
[c] Condensed Chemical Dictionary, Reinhold Publishing Corp., New York, ed. 3, 1942.

samples were spread over a fifteen-month period, it is believed that lots of different origin were involved. If so, the constancy of the values determined is quite interesting.

SUMMARY

The melting points and refractive indices at the melting point have been determined for many of the gums, waxes, and resins normally encountered in a pharmaceutical laboratory. These constants permit the ready identification of such samples.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

3,6,2′,4′-Tetrahydroxyflavone
3-Chloro-20-methylcholanthrene
6-Cyano-20-methylcholanthrene
Coriamyrtin
9-Phenyl-2,6,7-trihydroxy-3-fluorone
3,4,5-Trihydroxyanisole
Podophyllotoxin
Picropodophyllin
Podophyllic acid
Muscarine
Fructose-6-phosphate
Ketosuccinic acid
Uric acid riboside
Cadinene
D- and L-Erythrose
Adenine thiomethylpentoside
Pteroic acid
Phthionic acid
Fumigatin
Vanillyl amine

The Acute and Chronic Toxicity of 9-Aminoacridinepenicillin*,†

By DONALD C. BRODIE and ELIZABETH LOWENHAUPT‡

The LD_{50} of 9-aminoacridinepenicillin (9AAP) in mice is 562 mg./Kg. (subcutaneous) and 227 mg./Kg. (oral). The toxicity pattern of 9AAP resembles that of the acridine component. There is evidence that penicillin exerts a modifying influence on the acridine toxicity. Histopathologic studies reveal tissue necrosis at the site of injection and perihepatic damage involving the gall bladder.

THE RAPIDITY with which penicillin is eliminated from or destroyed in the body has directed attention toward improving the therapeutic usefulness of this antibiotic by methods designed to prolong or to enhance its activity. Prolongation has been obtained by the oil and wax combination of Romansky (1) and more recently by the use of procainepenicillin suspensions (2), some of which are further modified by the addition of aluminum monostearate (3, 4). Several workers have reported a prolongation of penicillin blood levels following the administration of other penicillin salts (2, 5, 6). *p*-Aminohippuric acid (7) and 4′-carboxyphenylmethanesulfonanilide (8, 9) interfere with the normal excretion of the drug, thereby prolonging effective penicillin concentrations in the blood. Recently, trace amounts of cobalt (10, 11) *in vitro* and *in vivo*, and vitamin K_5 *in vitro* (12) have been demonstrated to enhance the effectiveness of penicillin.

Daniels and Kumler (13) recently prepared a series of salts of penicillin with various amines, the one with 9-aminoacridine being of interest because of the antibacterial potency of the cation itself. This suggested that the salt might have a wider bacteriological spectrum than that of either the cation or the anion, or that the effectiveness of either component might be enhanced by the presence of the other component. Seeberg (14), in preliminary experiments in rabbits, observed a prolongation of penicillin blood levels when the 9-aminoacridinepenicillin was injected intramuscularly, and at the same time reported considerable tissue damage at the site of injection. These observations prompted a toxicological study of the compound.

9-Aminoacridinepenicillin (9AAP) is a bright yellow crystalline substance that is somewhat soluble in propylene glycol, glycerin, and alcohol, but practically insoluble in water. No loss in antibacterial activity was observed in samples stored in tightly stoppered dark-colored bottles at room temperature for several months, or in samples maintained at 37° and 90° for twenty-four hours. One milligram of 9AAP contains approximately 1000 units of penicillin and approximately 0.3 mg. of 9-aminoacridine (13).

ACUTE TOXICITY

9AAP.—The acute toxicity of 9AAP was studied in white mice, the group divided about equally as to sex, and each animal weighing approximately 20 Gm. The drug was dissolved in propylene glycol immediately before it was administered subcutaneously in the thigh area or by gastric instillation in concentrations that permitted the dose by either route to be contained in a volume of 0.1 to 0.2 cc. Control animals in each experiment received 0.2 cc. of propylene glycol. The mice receiving the intragastric dose were fasted for at least eight hours prior to the time of administration. LD_{50}'s and standard errors were determined by the method of Miller and Tainter (15) and are recorded in Table I.

Toxic symptoms of the drug were observed fifteen to twenty minutes after subcutaneous injection, although no deaths occurred for several hours. However, 63% of the deaths in the 100% mortality group occurred within twenty-four hours, with all deaths occurring within seventy-two hours. The general pattern of toxicity included muscular incoordination, general depression, and labored breathing. Death was probably due to respiratory failure since in the examined animals the heart beat continued after cessation of respiration.

When the compound was administered orally, the toxic and lethal effects were observed in much shorter time than when the drug was administered by injection. The 100% lethal dose (500 mg./Kg.) produced death in 6/7 animals in forty minutes. The symptomatology after oral administration was similar to that following subcutaneous injection: general depression, respiratory impairment, ataxia, and terminal clonic convulsions. In addition, muscular twitchings of the hind extremities and of the tail were a constant symptom, the latter manifestation and the convulsive seizures not being observed in the animals receiving the drug subcutaneously.

* Received May 2, 1949, from the College of Pharmacy and the School of Medicine, Department of Pathology, at the University of California Medical Center, San Francisco 22.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† This study was made possible by a research grant from the Cutter Laboratories of Berkeley, Calif.
‡ The authors wish to acknowledge the assistance of Professor W. D. Kumler and Mr. Edward C. Alpen for supplying the 9AAP used in the study, and of Mr. T. W. Schwarz for the stability data.

TABLE I.—THE LD_{50} OF 9-AMINOACRIDINEPENICILLIN AND OF 9-AMINOACRIDINE HYDROCHLORIDE (MICE)

Method of Administration	Compound	Expressed as	LD_{50}, Mg./Kg.	No. of Animals	Authority
Subcutaneous	9AAP	9AAP	562 ± 58.5	56	Present work
Subcutaneous	9AAP	9AAHCL	250	..	Present work
Intraperitoneal	9AAHCL	9AAHCL	70	..	Ungar and Robinson (16)
Subcutaneous	9AAHCL	9AAHCL	95	..	Rubbo (17)
Oral	9AAP	9AAP	227 ± 15.2	92	Present work
Oral	9AAP	9AAHCL	100	..	Present work
Oral	9AAHCL	9AAHCL	78 ± 17.0	47	Present work

9AAHCl.—When the study of 9AAP was concluded, there was evidence that the toxicity of 9AAHCL is modified when it is combined with penicillin (see Table I and Discussion). Furthermore, the chronic studies revealed organization of an extensive exudate arising probably in the gall-bladder area. Reference to this lesion could not be found in the literature of the acridines (18). Hence, it became expedient to determine the oral LD_{50} for 9AAHCL for the purpose of comparing it with that of 9AAP, and studying simultaneously the acute hepatic and renal toxicity. The nature of the experiment was identical with that described above for 9AAP. The mice were examined after death, or were sacrificed terminally, and sections for histologic study were made from the following organs: stomach, small bowel, liver with gall bladder and gall-bladder fossa, kidney, adrenal, pancreas, and spleen.

No changes were observed in the gastric or enteric mucosa. Likewise, no histologic changes were detected in the organs of those animals dying in three hours or less. At three hours some dilatation of small vessels in the cystic duct papillae was noted. There was slight interstitial edema and a few polymorphonuclear leucocytes were present about the vessels (Fig. 1). At eighteen hours a more extensive lesion was visible. It consisted of an exudate of fibrin with an occasional polymorphonuclear leucocyte and a few red blood cells on the liver capsule in the area of the gall bladder (Fig. 2). In one of these the gall-bladder wall itself was thickened by the presence of edema spaces and contained scattered polymorphonuclear leucocytes (Fig. 2). Dense collections of these cells were present about the cystic duct.

An additional change was seen in the kidney in 4 of the animals dying in eighteen to twenty-four hours. This consisted of deeply eosinophilic, homogeneous casts in the distal convoluted and collecting tubules, the glomerular tufts showing no changes (Fig. 5). This is similar to the "lower nephron nephrosis" following shock, burns, massive intravascular hemolysis, and other terminal states as described by Lucké (19). The tubular damage in the present experiments is interpreted as resulting from vascular collapse in the animals. Confirmation of this lesion was obtained in several mice through the observation of hemoglobin-tinged urine at the time of death. Acute hemolysis cannot be excluded as a cause but the former explanation seems probable since none of the organs were bile stained.

Fig. 1.—Acute toxicity of 9AAHCL in mice (oral) showing a portion of the cystic duct three hours after gastric instillation. Slight edema of papillae with dilatation of small vessels and scattered polymorphonuclear leucocytes in superficial areas characterize the early acute inflammatory reaction. Hematoxylin and eosin. 320×.

CHRONIC TOXICITY

9AAP.—A thirty-day semichronic toxicity experiment was conducted on a group of thirty 20-Gm. mice, 15 males and 15 females. The drug was injected subcutaneously in the thigh area in doses of 125 mg./Kg. in freshly prepared propylene glycol solutions adjusted so that the dose was contained in 0.1 to 0.2 cc. Daily injections were impracticable because of sloughing, hence the frequency of dosage was restricted to from three to six-day intervals. At the end of ten and twenty days, following 2 and 5 injections, respectively, 5 experimental and 2 control animals were sacrificed and autopsied for evidence of gross toxicity. The remaining 10 experimental and 3 control animals, after 7 injections, were sacrificed terminally (thirty days) and examined for gross changes, following which sections of liver and kidney were prepared for histologic examination. Three deaths occurred before termination of the thirty days. The animals in the ten-day sacrifice showed no toxic effects of the drug, and at the end of twenty days no gross abnormalities were apparent.

The only observable effect after injection was attributed to the hypertonicity of the solution and consisted of a general depression and localized twitching. Both manifestations were attributed to the propylene glycol since both appeared in the control animals and both have been reported previously for this solvent (20). All animals continued to gain weight during the course of the experiment.

Fig. 2.—Acute toxicity of 9AAHCL in mice (oral) showing the gall bladder eighteen hours after gastric instillation. A fibrino-hemorrhagic exudate appears on the serosal surface of the gall bladder and numerous polymorphonuclear leucocytes are present in the slightly thickened wall. The acute peri-cholecystic inflammatory reaction is somewhat more advanced than in Fig. 1. Hematoxylin and eosin. 160×.

Fig. 3.—Chronic toxicity of 9AAP in mice (subcutaneous) showing the organizing peritonitis involving the gall-bladder fossa. A portion of normal liver appears at the upper left below which is the cystic duct. This represents progression to a subacute stage of the process shown in Fig. 2. Hematoxylin and eosin. 160×

At thirty days, 4/10 experimental animals showed massive adhesions about the liver, obliterating completely the gall-bladder fossa and binding the liver capsule to the stomach wall, to loops of the large and small bowel, and to the diaphragm. Histologic examination revealed that the liver parenchyma was not involved, except for minimal extension of inflammatory cells in very superficial areas from the capsule along portal trabeculae. This capsular reaction consisted of numerous fibroblasts with areas of mature collagenous tissue between the liver capsule and the adhering organ: stomach, bowel wall, or diaphragm. In the latter case, inflammatory cells extended between muscle bundles to form dense clumps about the diaphragmatic lymphatics, while the walls of the adhering viscera were comparatively uninvolved. Plasma cells, lymphocytes, and to a lesser extent polymorphonuclear leucocytes, comprised the inflammatory cells. A few fibroblasts and scattered inflammatory cells were detected microscopically on the liver capsule of two mice that showed no gross changes. No histologic changes were observed in the kidneys or adrenals of the experimental animals. This organizing perihepatic peritonitis is shown in Figs. 3 and 4.

To insure that the hepatic lesions observed in the animals of the terminal sacrifice were attributable to the 9AAP, 20 additional control mice were autopsied, none of which showed perihepatic adhesions.

LOCAL TISSUE TOXICITY (9AAP)

Two 250-Gm. rats were injected subcutaneously with 9AAP in 0.2 cc. of propylene glycol. Doses of 15 mg./Kg. and 75 mg./Kg. were injected over the left and right thighs, respectively. Each animal served as its own control by receiving an injection of 0.2 cc. of propylene glycol over the left shoulder. At the end of twenty-four hours and again at forty-eight hours one animal was sacrificed and tissue sections were prepared from the injected areas. The histologic picture at the end of twenty-four hours was characterized by a large subcutaneous pocket of edema fluid at the site of injection with extension of tissue edema into the underlying skeletal muscle. Polymorphonuclear leucocytes were present in clusters between muscle bundles, although no muscle necrosis was demonstrable. Endothelial cells of small vessels were swollen and many of these vessels contained small fibrin thrombi. The control area at the end of twenty-four hours showed similar but less intense involvement: less edema, fewer inflammatory cells, and normal small vessels. The histologic responses at the two dosage levels were essentially the same. At the end of forty-eight hours, edema was widespread and a pocket of serum surrounded by necrotic muscle, fibroblasts, and polymorphonuclear leucocytes appeared at the site of injection. The area receiving only propylene glycol showed no pocket of serum and only a few fibroblasts and inflammatory cells were present (Fig. 6).

Intramuscular—Two 250-Gm. rats received injections of 9AAP in identical doses and prepared in the same manner as those for the subcutaneous injection. The drug was injected into the muscle mass of the upper hind leg. The inflammatory response was essentially the same as that described with subcutaneous injection, except that the locus was within the muscle and extended along surrounding fascial planes and nerve sheaths (Fig. 6). At the end of forty-eight hours some fibroblastic response was observed. The response to propylene glycol was of a similar type but of minor degree.

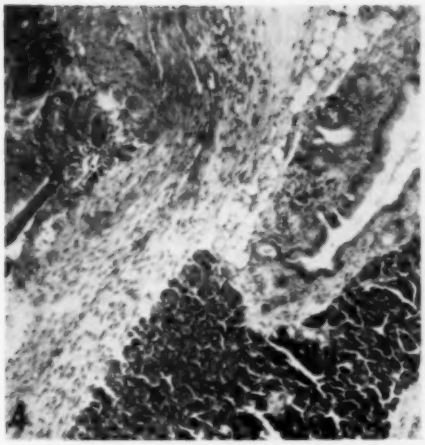

Fig. 4.—Chronic toxicity of 9AAP in mice (subcutaneous) showing a similar organizing exudate extending about the pancreas and cystic duct on the right, and the stomach wall on the left. Figures 3 and 4 are from different mice. Hematoxylin and eosin. 80×.

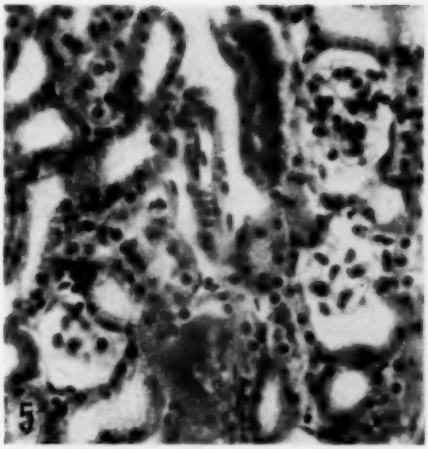

Fig. 5.—Acute toxicity of 9AAHCL (oral) showing the kidney of a mouse dying eighteen hours after receiving the drug. Two normal glomeruli are shown on the right. A loop of distal convoluted tubule filled with myohemoglobin appears at the left of the upper glomerulus, and a loop of collecting tubule containing similar material is shown in the lower center. This is the "lower nephron nephrosis" described by Lucké in a variety of conditions, including shock. Hematoxylin and eosin. 320×.

Local Application to Intact and Abraded Skin.—Two albino guinea pigs were shaved over both lateral areas and placed under observation for twenty-four hours to allow any localized traumatic effect to subside. A solution of 9AAP in propylene glycol, 30 mg./cc. (equivalent to 31,400 units of penicillin/cc. and 1 in 100 of 9-aminoacridine) was then applied with a camel's-hair brush to the right side of each animal, a small area of which had been scarified with a sharp instrument. Propylene glycol was applied to the left side, a small area of which had been similarly scarified. Both areas were massaged lightly and covered with gauze. At the end of twenty-four hours and again at the end of forty-eight hours 1 animal was sacrificed and tissue sections were made of the treated areas.

Neither 9AAP nor propylene glycol produced histologic changes in intact skin. In the scarified control areas the epithelium itself was intact, collections of fibrin focally replacing superficial keratin. The surface exudate contained scattered polymorphonuclear leucocytes, a few of which were present in the underlying derma. The small dermal vessels and the lymphatics showed no changes. In the scarified areas treated with 9AAP the inflammatory reaction was more intense. The entire epithelium was necrotic and large masses of fibrin were present over the surface. Dense collections of polymorphonuclear leucocytes appeared in this exudate and extended into the underlying derma. The small dermal vessels and lymphatic channels were dilated.

In order to observe the effect of 9AAP on human skin, 4 laboratory workers applied the drug (1 in 1000) in a washable ointment base to the skin of the volar surface of the forearm daily for five days with no demonstrable local reaction. The concentration in terms of penicillin was approximately 1000 units per gram; in terms of 9AAHCL, approximately 1 in 3000.

DISCUSSION

The toxicity of penicillin salts is accounted for largely by the toxicity of the cations (2, 21), hence the toxicity of 9AAP and of 9AAHCL should be similar. The above experiments confirm this thesis, at least in a qualitative way, since the general patterns of toxicity of the two compounds were similar. However, evidence obtained in this study indicates a quantitative modification in the toxicity of 9AAHCL when the compound is combined with penicillin. For example, the LD_{50} (subcutaneous injection) of 9AAP based on the acridine content of the salt and expressed as 9AAHCL is 250 mg./Kg. as compared with 70 or 80 mg./Kg. as reported by Rubbo (17) and Ungar and Robinson (16), respectively, for 9AAHCL, or a three- to four-fold modification. In the case of the oral LD_{50}, the difference between 100 mg./Kg. for 9AAP expressed as 9AAHCL and 78 mg./Kg. for 9AAHCL is less striking. This picture of a modifying influence of penicillin on acridine toxicity is in keeping with a similar observation of Alpen (22). He found that a solution of 9AAHCL was more toxic to leucocytes than one of 9AAP representing the same concentration of 9AAHCL.

Although this anomaly is not readily explained, one or more of the following factors may contribute to this phenomenon: (1) a lower degree of water solubility in the case of 9AAP, (2) a slow release of acridine from the salt *in vivo*, thereby retarding the establishment of critical toxic blood levels (3) the existence of the drug in solution as a colloidal electrolyte, thereby minimizing the toxicity of the drug by preventing concentrations of toxic ionized par-

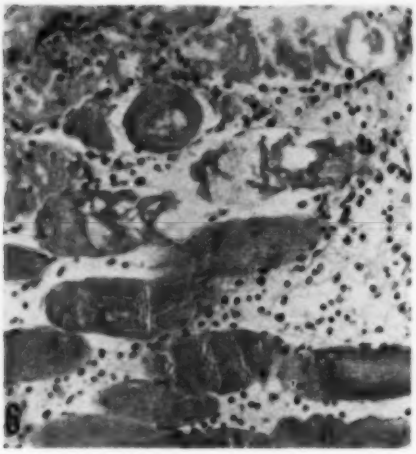

Fig. 6.—Local reaction of 9AAP in the rat showing tissue reaction forty-eight hours after intramuscular injection of the drug in propylene glycol. Necrotic muscle fibers and a small vessel, also necrotic, are shown in the upper half, the side nearest the injection site. Fibers separated by edema fluid appear below. The entire field is infiltrated by polymorphonuclear leucocytes. Hematoxylin and eosin. 160×.

ticles (13), and (4) a modification in the inherent toxicity of 9AAHCL.

One obvious explanation for the absence of the modifying influence of penicillin in 9AAP toxicity when the drug is administered orally is the hydrolysis of the salt in the gastric contents which liberates 9AA to exert its normal toxic effects. The quantitative similarity in the toxicity of 9AAHCL as expressed by the subcutaneous and oral LD_{50}'s is an interesting finding, since the rate of absorption of most agents is usually greater by subcutaneous than by oral routes of administration.

The general toxicity of the acridines is of two types: First, a local tissue necrosis with edema and acute inflammatory response (Fig. 6). This effect was observed in the experiments in which 9AAP was administered intramuscularly, subcutaneously, or applied to scarified skin. This is in agreement with the results of Rubbo (17) who observed an identical local tissue toxicity among members of a series of 16 acridine derivatives. Secondly, a systemic effect involving the liver in experimental animals. In this respect, the present results are not comparable to those of Meleney and Zau (23). These authors found central fat degeneration of liver lobules with generalized icterus and degeneration of the renal convoluted tubules in rabbits which died in six days after receiving 25 mg./Kg. of acriflavine by intravenous injection. No attempt is made to correlate the two sets of findings since the general experimental designs are so dissimilar. Clinically, acute yellow atrophy is reported (24) in man following the use of acriflavine as a urinary antiseptic.

However, the hepatic lesion resulting from the present study is a capsular and peritoneal reaction and does not represent parenchymal damage. The perihepatic distribution of scarring with massive involvement of the gall-bladder fossa is not to be explained by the healing of a liver injury of the type described previously (23, 24). A possible explanation is that the drug is excreted from the blood stream, at least in part, by way of the biliary passage. As it is concentrated in the gall bladder, the local necrotic and inflammatory effect is manifest in the gall-bladder wall and in the cystic duct, progressing through these tissues to the hepatic capsule. This is correlated with the early acute reaction seen in the cystic duct (Fig. 1). A focal peritonitis results, as seen in the animals dying from oral administration of 9AAHCL in eighteen to twenty-four hours (Fig. 2), and represented after thirty days with 9AAP by massive, organizing adhesions (Figs. 3 and 4). The renal lesion in these experiments appears to be a nonspecific result of terminal collapse rather than a specific renal injury caused by the drug.

CONCLUSION

The acute toxicity of 9AAP is qualitatively similar to that of the 9AA component, thereby restricting its therapeutic application to local surface rather than to systemic use. The local effects observed with 9AAP are in keeping with those reported previously for the acridines, although the systemic effects are not the same. 9AAP produces an acute cholecystitis progressing to massive acute perihepatic peritonitis, which in the chronic experiments organized to form adhesions to adjoining organs. This suggests that the compound is excreted at least in part by way of biliary passages. The only renal lesion observed, in contrast to previous reports of the acridines, is the "shock kidney" or "lower nephron nephrosis" described by Lucké and observed only in those animals dying acutely.

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3-Amino-4-hydroxybenzenearsonous Acid. III. Oxophenarsine Hydrochloride (Mapharsen). Decomposition Products*

By C. K. BANKS

Oxophenarsine hydrochloride (Mapharsen), is an antisyphilitic drug of importance in the therapy of spirochetal diseases. Since arsenicals having the 3-amino-4-hydroxyphenyl structure are known to decompose under unfavorable conditions, it was considered of interest to investigate the types of decomposition undergone by oxophenarsine as the hydrochloride and as the free base in solid form as well as solutions of these compounds at various *p*H levels. A study was made of the oxidative and hydrolytic decompositions of 3-amino-4-hydroxybenzenearsonous acid under a variety of conditions. The resultant products were determined quantitatively and investigated for possible toxicity. Various solid forms of the drug were also investigated.

OXOPHENARSINE Hydrochloride, U. S. P. (Mapharsen) is an important therapeutic agent in the treatment of treponemal diseases. Since toxic reactions are sometimes encountered in man from the use of arsenicals, the purity of this product has always been controlled by chemical and toxicity tests. Although these tests are rigid, the question has been raised as to the possible toxic effect of decomposition products which may result from prolonged shelf storage. Several conflicting statements on this subject may be found in the medical literature and in pharmacology textbooks, all of which are without experimental background. In order to answer this question properly, it was considered essential to investigate the possible decomposition routes and to isolate and identify the decomposition products.

Since the therapeutic agent is generally dispensed in ampuls as the solid chemical mixed with buffer and diluent and is administered in solution, it was necessary to consider the decomposition of both the solid agent and its solution at various *p*H levels. Pure oxophenarsine hydrochloride and its derivatives have been described previously (1, 2) and the essential pharmacological information is available in the literature (1–6). The methods of identification and isolation were standard techniques of arsenical chemistry (7, 8) and organic chemistry in general. 3-Amino-4-hydroxybenzenearsonous acid in solution was examined for decomposition between 6 *N* acidity and 6 *N* basicity, both aerobically and anaerobically, and between the temperature ranges of 0° and reflux temperatures. Solid 3-amino-4-hydroxybenzenearsonous acid (oxophenarsine) and anhydro-3-amino-4-hydroxybenzenearsonous acid hydrochlo ide (oxophenarsine hydrochloride, Mapharsen) were also examined at temperatures from 0° to 100° in the presence of and in the absence of oxygen.

EXPERIMENTAL

Chemically pure 3-amino-4-hydroxybenzenearsonous acid (1) which corresponded analytically to the theoretical values of total arsenic (8) and organically bound trivalent arsenic (7) within experimental limits, and pure 3-amino-4-hydroxybenzenearsonous cyclo-(1,3-As)-anhydride dihydrochloride (1) as determined by total arsenic, trivalent arsenic, ionic halogen, and acid titration, were used in most of the investigation. A few experiments were made with commercially available materials of U. S. P. or comparable quality.

Decomposition in Solution

1. Anaerobic, 6 *N* Hydrochloric Acid, Reflux.—Hydrochloric acid, A. R. (100 ml., 6 *N*), was refluxed for one-half hour and cooled under nitrogen. 3-Amino-4-hydroxybenzenearsonous acid (4.34 Gm.) was introduced and the solution heated under total reflux under nitrogen for three hours. A distillation head was attached and heating continued under nitrogen until 90 ml. of liquid was collected. The distillate was diluted to 1 L. Fifty-milliliter aliquots were neutralized with an excess of sodium hydroxide, made slightly acid with hydrochloric acid and then neutralized with sodium bicarbonate and a gram excess bicarbonate added. The aliquots were titrated with standard iodine (B. of S. arsenic trioxide standardized).

Standard iodine: 0.0505 *N*. Titration aliquots: 39.60, 39.54, 39.51, 39.59 ml. Arsenic trioxide recovered, 0.03996 equivalent or 99.88% of the arsenic available.

The residue in the flask was diluted to 25 ml. with water, neutralized to *p*H 6.5 (glass electrode) with sodium bicarbonate, and extracted with ten 25-ml. aliquots of ether. The ether was dried over anhydrous sodium sulfate at 0° and evaporated to yield 2.04 Gm. residue. The residue was a white, crystalline solid, m. p. 171–172°. Mixed m. p. with *o*-aminophenol unchanged. Recovery of *o*-aminophenol was 93.4% of the benzenoid residue.

* Received April 30, 1949, from Parke, Davis and Co., Detroit, Mich.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

A similar run with 3-amino-4-hydroxybenzenearsonous cycloanhydride hydrochloride (4.72 Gm.) resulted in the recovery of 99.72% of the arsenic as arsenic trioxide and 96.5% recovery of the organic residue as *o*-aminophenol.

2. Anaerobic, 6 *N* Hydrochloric Acid, 70°.—One hundred milliliters of 6 *N* hydrochloric acid was prepared as in Number 1. 3-Amino-4-hydroxybenzenearsonous acid (4.34 Gm.) was added to the acid at 70° in a water bath. The solution was maintained at total condensation under nitrogen and 10 ml. aliquots withdrawn at hourly intervals for five hours and the residue at the end of six hours. All aliquots were chilled immediately. The residue was treated as in 1. The aliquots were halved and the undecomposed organic arsenical determined by iodine titration (7) on one half and *o*-aminophenol recovered from the other half. The decomposition rate was calculated on the basis of undecomposed arsenical and *o*-aminophenol recovered (Table I).

TABLE I.—CALCULATED DECOMPOSITION RATES

Time, Hr.	Organic Arsenical, %	Decomposed, %	*o*-Aminophenol, %
1	68.1	31.9	30.6
2	47.2	52.8	51.4
3	32.4	67.6	68.0
4	22.2	77.8	75.9
5	15.6	84.4	83.2
6	...	100.0[a]	98.2

[a] Arsenic trioxide recovered, 99.2%.

3. Anaerobic, *p*H 2.1, Room Temperature.—A solution of 3-amino-4-hydroxybenzenearsonous cycloanhydride hydrochloride (1.2 Gm.) in 100 ml. of deoxygenized water was sealed in ten ampuls. At the end of five years the ampuls were examined, yielding the following results: *o*-aminophenol, 0.053 Gm., or 94.8% of the available benzenoid residue; organic arsenic, nil; arsenic trioxide, 0.498 mi.-eq., or 97.0% of the available arsenic.

4. Anaerobic, *p*H 6.5, Room Temperature.—A solution of 3-amino-4-hydroxybenzenearsonous acid which had been ampuled under conditions similar to Number 3 at *p*H 6.5 by Dr. A. B. Scott was available after fifteen years at room temperature. Large colorless crystals were apparent in the ampuls which proved to be *o*-aminophenol. Recovery of *o*-aminophenol was 92%. Arsenic trioxide analysis accounted for 99.72% of the arsenic.

5. Anaerobic, 6 *N* Sodium Hydroxide, Reflux.—An oxygen-free solution of 6 *N* sodium hydroxide (100 ml.) and 3-amino-4-hydroxybenzenearsonous acid (6.5 Gm.) was refluxed under nitrogen for four hours. After neutralization the condenser was reversed and the steam-volatile materials removed by steam distillation. The distillate was extracted with ether and the solid collected weighed 2.47 Gm., m. p. 173°. Mixed m. p. with *o*-aminophenol was unchanged. The *o*-aminophenol recovered was equivalent to 90.6% of the available benzenoid residue. Titration of aliquots of the residue in the reaction flask indicated 24.6 milliequivalents of arsenic trioxide, or 98.4% of the available arsenic.

6. Anaerobic, *p*H 8.0, Room Temperature.—3-Amino-4-hydroxybenzenearsonous acid (1.2 Gm.) was dissolved in 100 ml. of oxygen-free water adjusted to *p*H 8 with sodium hydroxide. The solution was ampuled in 10 ampuls and held for five years. In spite of all precautions, some oxygen was admitted as evidenced by the development of color. The amount of color was not excessive, and at the end of five years 96.8% of the arsenic was present as inorganic arsenic and 88.2% of the organic residue was recovered as *o*-aminophenol.

7. Aerobic, Neutral, Hydrogen Peroxide.—A suspension of 3-amino-4-hydroxybenzenearsonous acid (10.9 Gm.) in 100 ml. of water was treated with an excess of hydrogen peroxide with gentle warming. A light pink color was generated, and at the end of the reaction sufficient hydrochloric acid was added to dissolve the product, the solution treated with charcoal (Darco G-60) and filtered. Addition of more hydrochloric acid to the filtrate yielded 12.7 Gm. of 3-amino-4-hydroxybenzenearsonic acid hydrochloride hemihydrate, representing 88% conversion.

8. Aerobic, *p*H 4, Iodine.—In a previous publication (7) it was shown that 3-amino-4-hydroxybenzenearsonous acid reacted quantitatively with iodine between *p*H 2 and *p*H 5. The reaction product, isolated at *p*H 4, proved to be 3-amino-4-hydroxybenzenearsonic acid.

9. Aerobic, *p*H 6.5, Room Temperature.—A solution of 3-amino-4-hydroxybenzenearsonous acid was prepared as in Number 4. Oxygen was passed through the solution for six days at room temperature. A control solution was kept under nitrogen to compensate for hydrolytic decomposition. At the end of six days, the oxygenated solution contained a mass of black material and the solution was dark brown, while the anaerobic solution was colorless. The hydrolytic decomposition in the anaerobic solution was determined as previously described. The oxygenated solution was split into several aliquots and examined. The organically bound trivalent arsenic determinations are presented in Table II.

TABLE II.—ORGANICALLY BOUND TRIVALENT ARSENIC DETERMINATIONS

Solution	% As (III-org.) of Original Value[a]
Anaerobic	90.9
Oxygenated, untreated	89.0
Oxygenated, filtered	88.9
Oxygenated, charcoaled	88.9
Oxygenated, supernatant from centrifuging	89.2
Oxygenated, supernatant from centrifuging after charcoaling	89.4
Precipitate	0.0

[a] End point determined potentiometrically.

An aliquot was decolorized and the organic arsenical remaining isolated. Based on known solubilities and recoveries, the unchanged 3-amino-4-hydroxybenzenearsonous acid recovered accounted for 88.6% of the starting material.

Examination of an aliquot gave no evidence of the arsonic acid, 3-amino-4-hydroxybenzenarsonic acid.

The insoluble solid proved to be free of organic and inorganic arsenic. This product contained at least two constitutents which were identified as 2-amino-3-isophenoxazone and 2-hydroxy-3-isophenoxazone (9–13), known oxidation products of *o*-aminophenol.

10. Aerobic, *p*H 2, Room Temperature.—The experiment described under Number 9 was repeated with a solution prepared as in Number 3. The aerobic solution formed a dark, insoluble product as in Number 9. No essential difference in the amount of organic arsenical was noted between the aerobic and anaerobic solutions. The dark material was found to be a mixture of oxidation products of *o*-aminophenol.

11. Aerobic, *p*H 9, Room Temperature.—A solution of 4 Gm. of 3-amino-4-hydroxybenzenearsonous acid in 100 ml. of water was adjusted to *p*H 9 with sodium hydroxide and oxygen passed through the solution for five days. A control solution was maintained under nitrogen. There was considerable evidence of decomposition. The insoluble materials were filtered off and the filtrate was assayed for undecomposed arsenical. The oxygenated solution contained 72.4% undecomposed arsenical as compared with 87.4% in the anaerobic control solution. The insoluble fraction contained arsenic in organic combination. The arsenic content of the residue varied considerably in the range 20–25%, and nitrogen values were from 3.5% to 12%. The decomposition product was about 15% of the starting material. After benzene and petrolic ether extractions, the brick-red residue appeared to consist of two fractions, both containing about 23% arsenic. Further separation by fractional precipitation from acid solution yielded two substances of nearly the same properties.

Substance X: Brick-red, no m. p. *Calcd.* for $C_{12}H_8AsN_2O_4$: As, 23.40%; N, 8.75%. Found: As, 23.36%; N (Kjeldahl), 8.52%.

Substance XI: Brick-red, no m. p. *Calcd.* for $C_{12}H_8AsNO_5$: As, 23.33%; N, 4.36%. Found: As, 23.34%; N, 4.38%.

Some nonarsenated decomposition products of *o*-aminophenol were also isolated.

Decomposition of Solid

12. Oxophenarsine Hydrochloride, Anaerobic, Anhydrous, 100°.—3-Amino-4-hydroxybenzenearsonous cyclo-(1,3-As)-anhydride dihydrochloride (20 Gm.), containing less than 0.05% volatile constituents as determined by the U. S. P. method for oxophenarsine hydrochloride, was sealed under nitrogen in 20 ampuls of 1 Gm. each. Fifteen ampuls were heated at 100° while the remaining five were held as controls. After twenty-four hours the ampuls showed little or no evidence of decomposition, after forty-eight hours some discoloration, after ninety-six hours a definite darkening. The ampuls were removed after five days and examined. The product was still completely soluble in water.

Organic Arsenical.—Trivalent organic arsenical titration yielded values of 85.6, 84.8, 86.1% of the original value. The organic arsenical was isolated and proved to be unchanged. Isolation accounted for 84.8, 85.2, 83.9% of the starting arsenical.

Nonarsenated Organic Residues.—Ether extraction of the contents of one ampul removed 0.014 Gm. of a somewhat discolored material, which was largely *o*-aminophenol. The contaminants were a small amount of a brown nonarsenical residue and traces of organic and inorganic arsenic. This quantity accounted for 3.1% of the decomposition as *o*-aminophenol.

Alcoholic extraction with absolute *n*-propanol removed an average of 0.085 Gm. per Gm. of starting material. The ether-soluble fraction contained 0.015 Gm. as before, while the residue of 0.07 Gm. was identified as being largely *o*-aminophenol hydrochloride. The nonarsenated residue accounted for nearly 14% decomposition to *o*-aminophenol derivatives.

Inorganic Arsenic.—An attempt to identify the approximately 15% inorganic arsenic was made. Vacuum evacuation at 1 mm. through a dry-ice trap using a 50° heating bath and collection in the trap immersed in an ether–dry-ice mush resulted in the volatilization of approximately 2% to 3% of the total arsenic. Since chlorides were present in the distillate, the volatile arsenic was probably present as arsenic trichloride.

Efforts to isolate the remainder of the arsenic failed. Inasmuch as arsenic trioxide is difficult to separate from the other compounds identified, this was not unexpected.

A similar experiment at 70° showed such slight decomposition in one week as to be of no value in this study.

13. Oxophenarsine, Anaerobic, Anhydrous, 100°.—A sample of analytically pure 3-amino-4-hydroxybenzenearsonous acid (99.97% by total arsenic, 99.98% by trivalent arsenic, volatile content 0.03%) was ampuled in 2-Gm. quantities under nitrogen. A number of ampuls were retained as controls while others were heated at 100°. After twenty-four hours the character of the crystalline material was notably altered and colorless; glistening platelets started to collect in the necks of the ampuls. At the end of a week those ampuls which had not fractured (due to internal pressure) were removed from the oven and examined. The amount of undecomposed arsenical as determined by titrimetric analysis was essentially negligible. The platelets formed were identified as *o*-aminophenol and ether extraction accounted for 99.8, 99.6, 99.3% of the organic residue. The residue after extraction of *o*-aminophenol was analyzed for arsenic trioxide and was found to contain 99.87, 99.91, 99.78% of the original available arsenic.

14. Oxophenarsine Hydrochloride, Anaerobic, Anhydrous, Room Temperature.—The control ampuls of Number 12 were held in storage at room temperature. The samples were examined yearly. At the end of five years the decomposition amounted to about 1% and the experiment was discontinued.

15. Oxophenarsine, Anaerobic, Anhydrous, Room Temperature.—The control ampuls of Number 13 were held at room temperature and examined at yearly intervals. At the end of five years the decomposition amounted to about 1%, the products of decomposition being *o*-aminophenol, arsenic trioxide, and water in quantitative amounts.

16. Oxophenarsine Hydrochloride, Anaerobic, Hemihydrate, 100°, 70°, and Room Temperature.—A sample of bis-(3-amino-4-hydroxybenzenearsonous) anhydride dihydrochloride meeting U. S. P. specifications for oxophenarsine hydrochloride except for volatile content (3.59%) was ampuled in 2-Gm. quantities under nitrogen. One group of ampuls was heated at 100°, a second group at 70°, and a third was held at room temperature.

At 100°, decomposition was evident in eight hours and liquefaction was noticeable at twenty-four hours. After forty-eight hours, the ampuls were removed.

Titration for organic trivalent arsenic, 2.46%; inorganic arsenic, 97.38%; *o*-aminophenol recovered, 93.6%. At 70° the same decomposition process was noted except that over a month was required for essentially complete decomposition. At room temperature, the ampuls did not show serious deterioration after six months, and at the end of three years the decomposition was of the order of 3%. The decomposition products were the same as those isolated in the 100° test.

3-Amino-4-hydroxybenzenearsonous acid hydrochloride (7.08% volatile content) was examined similarly. Decomposition was more rapid but the products were the same.

17. Oxophenarsine Hydrochloride, Aerobic, Anhydrous, 100°, 70°, and Room Temperature.—Samples of 3-amino-4-hydroxybenzenearsonous cycloanhydride hydrochloride were prepared as in Number 12 except that the ampuls were filled with oxygen before sealing. The volume was such that the oxygen was present in the ratio of one molecule of oxygen for eight of arsenic. The samples were divided into three lots, one of which was placed in a 100° oven, a second in a 70° oven, while the third was kept at room temperature. The samples at 100° showed a slight darkening in twenty-four hours, more at ninety-six hours, and serious blackening in five days. Assays of samples after five days indicated 83.8, 84.2, 85.7% undecomposed material. Decomposition products were identified as at least three nonarsenated oxidation products of *o*-aminophenol. The samples at 70° began to discolor on the fourth day and were tan in color in two weeks. However, the undecomposed portion was 98.54, 98.21, 97.67% of the starting samples. Room temperature samples showed only slight darkening after five years and the average assay indicated less than 1.6% decomposition.

18. Oxophenarsine Hydrochloride, Aerobic, Hydrated, 100°, 70°, and Room Temperature.—The materials used in Number 16 were also ampuled in the presence of oxygen. The hemihydrate samples began to liquefy at 100° in eight hours with considerable blackening. After forty-eight hours, the ampuls were examined and found to be completely decomposed. No organic trivalent arsenic was found and the residue consisted of arsenic trioxide, arsenic trichloride, *o*-aminophenol hydrochloride, and at least two oxidation products of *o*-aminophenol. The 70° samples required longer for decomposition but the resultant products were the same. The room temperature samples after three years contained 87.36% undecomposed material, the remainder being inorganic arsenic (principally trioxide), a slight amount of *o*-aminophenol and the oxidation compounds identified previously.

The hydrate salt decomposed more rapidly, but the products of decomposition were the same.

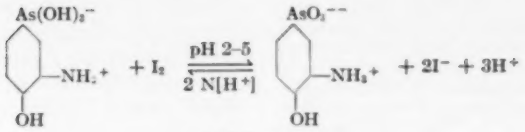
DISCUSSION

Decomposition in Solution.—The decomposition of oxophenarsine hydrochloride (I) and related compounds (II, III, IV, V, and XII) in solution was independent of the particular arsenical form used and was dependent only on the *p*H, temperature, concentration, and oxygen tension of the solution. This was expected from general knowledge of the behavior of arsenicals, although it has been alleged that the salt grouping and exact nature of the arsenic group could influence the ion structure in solution (14). As has been shown previously (1) oxophenarsine hydrochloride (I) (3-amino-4-hydroxybenzenearsonous cyclo-[1,3-As] anhydride dihydrochloride), the corresponding hemihydrate (II) (bis-[3-amino-4-hydroxybenzenearsonous] anhydride dihydrochloride or bis-[As-hydroxy-3-amino-4-hydroxyphenylarsyl] oxide dihydrochloride), and the hydrate (III) (3-amino-4-hydroxybenzenearsonous acid hydrochloride), as well as the corresponding dichloroarsine, dichlorophenarsine hydrochloride (XII) ([3-amino-4-hydroxyphenyl]dichloroarsine hydrochloride), and free base, oxophenarsine (V) (3-amino-4-hydroxybenzenearsonous acid), all yield the common ion, (3-protoamino-4-hydroxyphenyl)trihydroxyarsenic-(+3)-ate (IV), when dissolved in water above 5° and adjusted so as to be between *p*H 1 and *p*H 7. Below *p*H 1 in hydrochloric acid the trichloroarsenic-(+3)-ate ion (XII) becomes a part of the aqueous system and, at low temperatures, the chlorodihydroxyarsenic-(+3)-ate ion (XIII) can be detected. As long as oxygen was removed from the system, the decomposition products appeared to be due to the same or similar mechanism regardless of *p*H. Thus, in 6 *N* acid, at *p*H 2, *p*H 6.5, *p*H 8, and 6 *N* base and at temperatures ranging from room temperature to over 100°, the organic portion of IV was converted to *o*-aminophenol (VI) or its salts, while the arsenic was demonstrated to be as arsenic trioxide, arsenic trichloride, or sodium arsenite. No evidence for the formation of tris-(3-amino-4-hydroxyphenyl)arsine (XIV) or bis-(3-amino-4-hydroxyphenyl)hydroxyarsine (XV) was found although Ehrlich and Bertheim (15) identified tris-(4-aminophenyl)arsine from the hot acid decomposition of 4-aminobenzenearsonous acid and Christiansen (16) isolated what he thought to be XV, as well as *o*-aminophenol and arsenic trioxide, from the hot acid decomposition of 3-amino-4-hydroxybenzenearsonous acid. More recently Blicke and co-workers (17) have prepared all of the possible disproportion products of IV (XIV, XV, and others) by independent methods, and the properties of the compound isolated by Christiansen do not agree with any of these compounds and agree more nearly with those of impure IV. The quantitative recoveries of Experiments 1, 2, 3, and 4 as well as nearly quantitative recoveries in 5 and 6 indicate a hydrolytic cleavage of arsenic with the insertion of a hydrogen for arsenic. This cleavage is a common feature in many arsenicals, particularly trivalent arsenic-substituted phenols.

The decomposition IV to VI appears to be irreversible since no equilibrium state was achieved, even after fifteen years. Although arsenic trichloride and arsenic acid have been used as arsenating agents, no record of arsenation with arsenic trioxide has been found in a survey of the literature, making such a conclusion compatible with known chemical behavior.

The introduction of possible oxidation as an additional factor altered the appearance of the decomposition. When the oxidizing agent was iodine, the arsenic was oxidized quantitatively according to equation I.

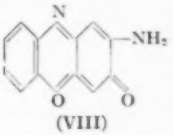
The reverse reaction has also been demonstrated to be quantitative (7). No oxidation of the *o*-aminophenol residue has ever been noted in this reaction. Similarly, hydrogen peroxide oxidizes IV to the ar-



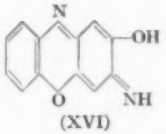
EQUATION I

senic acid and, while the evidence is not as conclusive as with iodine, it appears to be possible to carry out this oxidation without appreciable nuclear oxidation. Also, in the light of the other oxidations, any apparent nuclear oxidation may be due to oxidation of free *o*-aminophenol. While both iodine and hydrogen peroxide oxidize IV rapidly, under comparable conditions of time, temperature, and *p*H, oxygen and air have little or no effect on the arsenical.

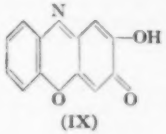
The effect of oxygen and air on prolonged exposure is profound, the solution becoming yellow, then brown, followed by the formation of dark red, brown, or black insoluble materials. The *p*H of the solution also influences the appearance and the identity of the oxidation products. The action of oxygen below *p*H 7 appeared to be uniform with respect to the types of products formed. In Experiment 9 it was evident that the insoluble oxidation product was not produced by direct oxidation of the arsenical and the parallel decrease in organic arsenical in the anaerobic and aerobic jars indicated that the oxidation was independent of the organic arsenical and dependent only on the hydrolytic rate. *o*-Aminophenol has been demonstrated to yield 2-amino-3-isophenoxazone (VIII) and 2-hydroxy-3-isophenoxazone imino (XVI) by Kehrmann and Mattisson (9) on air oxidation in hot water. VIII was identified as one product and XVI was undoubtedly also present since 2-hydroxy-3-isophenoxazone (IX), the hydrolytic product (13) of XVI, was also present. In addition to these three products, there was also evidence (10–12) of the presence of triphendioxazin (XVII). Since the literature indicates very little as to the relative ratio of VIII, IX, XVI, and XVII which may be produced, the possible influence of the arsenic hydrolysis on oxidation cannot be established. However, the relatively large amounts of VIII suggest that during hydrolysis an electron-deficient radical is formed which undergoes rapid oxidation. Since all of the products of oxidation require some unoxidized *o*-aminophenol in their formation, it is apparent that some conversion to *o*-aminophenol and subsequent oxidation and combination exist. It is apparent that, under *p*H 7, 3-amino-4-hydroxybenzenearsonous acid is stable toward oxygen and air and is furthermore not affected by the oxidized form of *o*-aminophenol (VII).



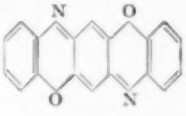
(VIII)



(XVI)



(IX)



(XVII)

In alkaline solution the situation is changed and the principal products of oxidation contained arsenic. The structures of these products (X and XI) were assigned by analogy to VIII and IX and the arsenic and nitrogen assays agree very well. The oxidation reaction above *p*H 7 appears to be highly complex and while a comparable rate study was not made, it was suggestive that more material decomposed at *p*H 9 in five days than at *p*H 6.5 in six days. The mechanism is apparently connected with the hydrolysis since the arsenic appears to enter the final product through IV rather than an oxidized form and it is the combination of the oxidized quinone intermediate (VI) with the arsenical which resulted in X and XI. A quantitative study of the oxygen uptake is being made in conjunction with toxicity measurements in another laboratory (18).

Decomposition of Solid.—The anhydrous solid chemical was extremely stable, showing no evidence of measurable decomposition in twenty-four hours and only about 15% in five days at 100°. The decomposition products were practically the same as those found in acidic solutions, *o*-aminophenol when anaerobic and isophenoxazones in the presence of oxygen. The decomposed arsenical was converted to inorganic arsenic. At temperatures below 100° the amount of decomposition was insignificant even though the aerobic samples showed appreciable color, while at room temperature the decomposition was scarcely measurable. The presence of water hastened the decomposition as evidenced by the hemihydrate (II) and hydrate (III) in Experiment 16. Similarly oxophenarsine free base (V) was less stable to heat than the hydrochloride. There was definite evidence that temperatures around 100° caused more profound decomposition in the free base (V) than was evidenced in much longer periods at lower temperatures.

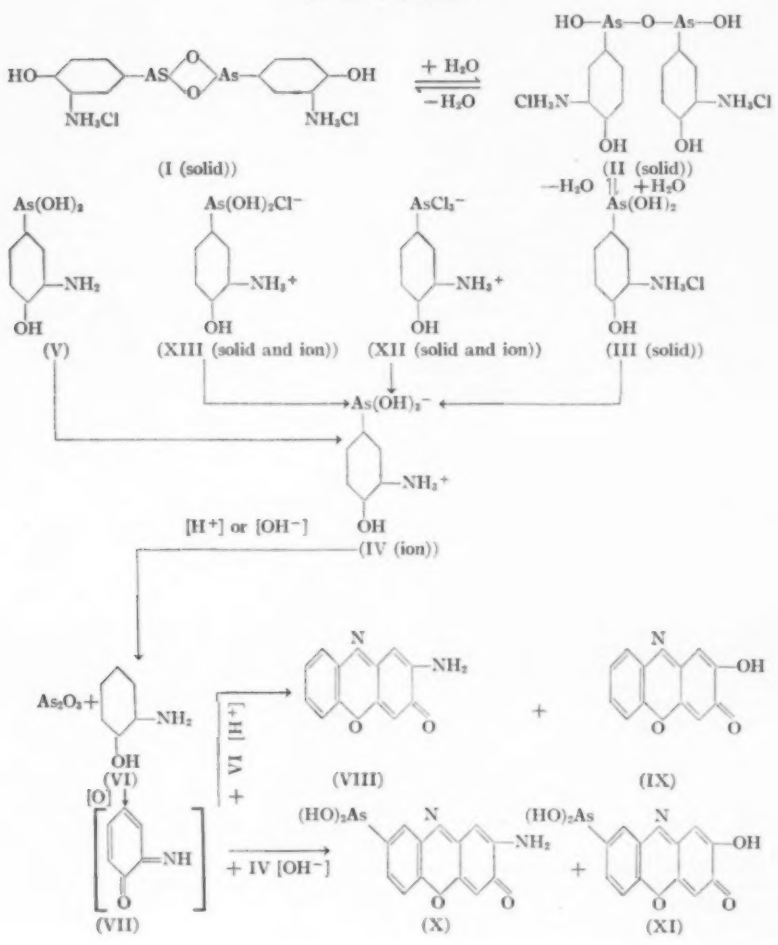
Toxicity.—The various products isolated were tested by Dr. O. M. Gruhzit of these laboratories for intravenous toxicity to albino rats. *o*-Aminophenol and its oxidation products were relatively nontoxic as compared with oxophenarsine hydrochloride. Similarly arsenic trioxide, compared on an equivalent arsenic basis, is less toxic. The isophenoxazone arsenicals were obtained in very small amounts and not enough material was available for a critical study, but they were no more toxic to rats (in terms of mg. As/Kg.) than was oxophenarsine hydrochloride. Consequently, partially or even completely decomposed ampuls would be less toxic than the original contents. This was verified on a large number of commercial ampuls which had decomposed to the extent of 5% to 10%.

SUMMARY

1. Solid oxophenarsine hydrochloride is extremely stable when anhydrous, the amount of decomposition in five years being scarcely perceptible. The stability decreases with increasing water content. Oxygen does not react with oxophenarsine hydrochloride but colored oxidation products may result due to the action of oxygen on hydrolytic products.

2. Solid oxophenarsine hydrochloride (hydrated) and oxophenarsine are decomposed to *o*-aminophenol and inorganic arsenic compounds in the absence of oxygen. In the presence of oxy-

CHART OF REACTIONS



gen a part of the *o*-aminophenol is converted to the extremely reactive iminoquinone intermediate which reacts with more of the aminophenol to produce nonarsenated isophenoxazones.

3. Solutions of oxophenarsine decompose slowly in the cold, more rapidly with heat to *o*-aminophenol and inorganic arsenite. In the absence of oxygen no other products are formed. In the presence of oxygen, *o*-aminophenol is oxidized and below *p*H 7 condenses with unoxidized *o*-aminophenol to yield nonarsenated isophenoxazones. Above *p*H 7, oxygen oxidizes the *o*-aminophenol produced to a postulated iminoquinone which combines with unhydrolyzed oxophenarsine to give arsenosoisophenoxazones.

4. The decomposition products of oxophenarsine hydrochloride we e found to be less toxic than the drug itself.

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3-Amino-4-hydroxybenzenearsonic Acid. IV. Oxophenarsine Hydrochloride (Mapharsen). Decomposition Rate*

By C. K. BANKS†

In the solid form the rate of decomposition of oxophenarsine hydrochloride was found to be dependent on the moisture content and on temperature. The extent to which decomposition occurred was limited by the quantity of water available and was stoichiometrically equivalent in amount. Oxygen had no influence on the decomposition rate but altered the final products of decomposition. The rate of decomposition of solutions of oxophenarsine hydrochloride was altered by temperature and *p*H but was independent of oxygen. The nature of the final decomposition products was altered by oxygen. Solutions of the drug are adequately stable for therapeutic use at room temperature for at least three days provided the solution is protected from oxygen.

IN A PREVIOUS paper (1) the possible effect of the decomposition of oxophenarsine hydrochloride on the toxicity of the drug was considered. It was found that the decomposition products were less toxic than the drug and that oxophenarsine hydrochloride was quite stable. Since color was noticed in older samples, it was felt that a quantitative examination of the decomposition rate under controlled conditions would indicate more clearly the stability of the drug with relation to volatile content, initial impurities, and conditions of ampuling and storage. It was also hoped that some relationship could be established between physical appearance, accelerated breakdown tests, useful life, and age.

Since the decomposition in solution was similar to that of the solid, a similar stability study was undertaken to establish the decomposition rate in aqueous media, with particular reference to the possible length of time a solution would be satisfactory for clinical use.

The chemistry of arsenicals of this type has been described previously (1–3) as well as the principle methods of investigation (4–6). The materials for the investigation were either special research samples or commercial material. In

* Received April 30, 1949, from Parke, Davis and Co., Detroit, Mich.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† The author wishes to acknowledge the technical assistance of John Controulis and Donald F. Walker in several studies of solutions and the helpful suggestions of Dr. L. A. Sweet and Messrs. F. E. Willson, F. A. Maurina, and J. S. Malcolm.

most experiments the data have been grouped from several separate studies to yield a composite picture over a wider range of conditions than would be possible in a single experiment.

The criteria used to determine the degree of breakdown were several. The samples were assayed to determine per cent oxophenarsine hydrochloride which was undecomposed; they were judged on the basis of color of solid and behavior in solution; and they were studied for heat stability.

EXPERIMENTAL

1. Stability of Pure Chemical.—Lots of analytically pure 3-amino-4-hydroxybenzenearsonous cyclo-(1,3-As) anhydride dihydrochloride were ampuled *in vacuo*. A volatile determination was made on each lot and varying numbers of ampuls were stored at 20, 30, 40, 50, 60, and 70°. The samples were examined after three months, six months, one year, two years, three years, and five years. It was found that the data could be grouped into four major categories, based on the volatile content of the chemical. The first consisted of those lots which were essentially anhydrous (less than 0.05% volatile content); the second, those having approximately 0.5% volatile content; the third, those of about 1% volatile; and the fourth, those having around 1.5% volatile content. The composite results for the first group are listed in terms of average per cent undecomposed chemical in Table I, the second group in Table II, the third group in Table III, and the fourth group in Table IV.

2. Stability of Commercial Material.—A number of lots of the commercial form of oxophenarsine hydrochloride (Mapharsen), which is a mixture of oxophenarsine hydrochloride U. S. P., sodium carbonate, sucrose, and ascorbic acid, were subjected to an initial inspection procedure to determine total arsenic, oxophenarsine hydrochloride, color, appearance and color of solution, volatile content, and heat stability. The lots were filled into ampuls and sealed (one half sealed "as is" and the other half with the air largely displaced by nitrogen). The lots so examined were chosen to fit into three groups. The first group consisted of standard materials which differed from the normal lots by having an abnormally low volatile content (below 0.05%), the second group were normal production samples meeting U. S. P. limits (<0.5% volatile, average 0.26%), while the third group were lots which were standard except for a high volatile content (>0.5% average 0.67%). Since the results were consistent within each group, the data were averaged for each group for the purpose of simplification. The various lots were stored at room temperature (20° to 35°) and examined thoroughly after three months, six months, a year, two years, three years, and four years. The criteria observed were per cent decrease in oxophenarsine hydrochloride, color, appearance of solution, heat stability at 70°, and appearance in solutions of heated samples. Color changes in solid samples were noted by comparison with graded color backgrounds. Solutions were examined for color change in a colorimeter and for haze by nephelometric methods. The codes used for recording data are as follows:

Color of Solid: 0 = colorless against a white plate, 1 = off-color on a white plate but insufficient for color classification, 2*b* = first perceptible shade of buff, 2*y* = first perceptible shade of yellow, 3*b* = perceptible tan, 3*y* = perceptible yellow, 4*b* = tan, 4*y* = yellow, 5 = definite darkening or black specks. Ratings of 4 and 5 would not be acceptable by U. S. P standards.

Solution: Two designations were used, the first designated color using the code above, the second classified appearance as follows: 0 = clear to the same extent as the water used to prepare the solution; 1 = perceptible increase in Tyndall effect under magnification; 2 = faintest haze in a Tyndall beam without magnification; 3 = limit of U. S. P. tolerance for solutions; 4 = haze greater than allowed by U. S. P.; 5 = floaters and large debris. The composite data are listed in Table V.

TABLE I.—STABILITY OF OXOPHENARSINE HYDROCHLORIDE (ANAEROBIC, ANHYDROUS—VOLATILE CONTENT <0.05%)

Temp.,°	% Undecomposed After[a]					
	3 Mo.	6 Mo.	1 Yr.	2 Yr.	3 Yr.	5 Yr.
20	99.97	99.95	99.88	99.76	99.63	99.47
30	99.92	99.87	99.75	99.57	99.42	99.21
40	99.72	99.48	99.01	98.84	...	98.69
50	98.90	98.67	98.65	...	...	...
60	98.70	98.65	...	...	...	...
70	98.61	98.58	...	...	...	98.42

[a] All samples colorless.

TABLE II.—STABILITY OF OXOPHENARSINE HYDROCHLORIDE (ANAEROBIC, VOLATILE CONTENT <0.5%)

Temp.,°	% Undecomposed After[a]					
	3 Mo.	6 Mo.	1 Yr.	2 Yr.	3 Yr.	5 Yr.
20	99.89	99.81	99.65	99.28	98.76	97.91
30	99.78	99.62	99.25	98.72	98.22	97.08
40	99.34	98.82	96.25	...	...	...
50	96.70	...	...	...	...	...
60	95.35	...	...	...	...	...
70	95.21	93.48	93.42	...	...	...

[a] All samples colorless.

TABLE III.—STABILITY OF OXOPHENARSINE HYDROCHLORIDE (ANAEROBIC, VOLATILE CONTENT <1.0%)

Temp., °	% Undecomposed After[a]					
	3 Mo.	6 Mo.	1 Yr.	2 Yr.	3 Yr.	5 Yr.
20	99.76	99.54	99.06	98.28	97.35	95.55
30	99.62	99.29	98.65	97.31	96.01	94.17
40	99.31	98.58	97.31	...	...	...
50	97.87	...	...	...	...	...
60	91.78	...	...	...	...	...
70	89.25	...	...	...	...	..

[a] All samples colorless.

TABLE IV.—STABILITY OF OXOPHENARSINE HYDROCHLORIDE (ANAEROBIC, VOLATILE CONTENT <1.5%)

Temp., °	% Decomposition After[a]					
	3 Mo.	6 Mo.	1 Yr.	2 Yr.	3 Yr.	5 Yr.
20	99.61	99.24	98.60	97.12	95.77	92.65
30	99.31	98.72	97.24	94.71	92.08	89.21
70	81.21	79.67	...	...	...	...

[a] All samples colorless.

TABLE V.—STABILITY OF COMMERCIAL OXOPHENARSINE HYDROCHLORIDE (MAPHARSEN) IN AMPULS

Age	Test Results				[a]N. I. H.—Heat Test—70°			
	Oxophenarsine Hydrochloride Loss, %	Color of Solid	Solution		Time Hr.	Color of Solid	Solution	
			Color	Appearance			Color	Appearance
		(*a*) Volatile Content <0.05%, Under N_2						
0	0.0	0	0	0	168	1	1	0
6 mo.	0.1	0	0	0	168	1	1	0
1 yr.	0.2	0	0	0	168	1	1	0
2 yr.	0.5	1	0	0	168	2*b*	1	1
					144	1	1	1
3 yr.	1.1	1	1	0	144	2*b*	1	1
					120	1	1	1
4 yr.	1.5	1	1	1	120	1	2*a*	1
					96	1	1	1
		(*b*) Volatile Content <0.05%, Under Air						
0	0.0	0	0	0	120	1	1	1
					96	0	1	0
6 mo.	0.1	0	0	0	96	1	1	0
1 yr.	0.2	0	0	0	96	1	1	1
					72	0	1	0
2 yr.	0.5	0	0	0	96	1	1	1
					72	1	1	0
3 yr.	1.2	1	1	0	96	2*b*	3*y*	3
					72	1	2*y*	1
					48	1	1	1
4 yr.	1.7	1	1	1	96	3*b*	3*y*	3
					72	2*b*	2*y*	2
					48	1	2*y*	1
		(*c*) Volatile Content <0.5%, Under N_2						
0	0.0	0	0	0	168	2*b*	2*y*	1
					144	1	1	1
					120	1	1	0
6 mo.	0.4	0	0	0	144	1	1	1
					120	1	1	0
1 yr.	1.0	0	0	0	144	2*b*	2*y*	2
					120	1	2*y*	1
					96	1	1	0
2 yr.	2.1	1	1	0	120	2*b*	2*y*	1
					96	1	2*y*	1
					72	1	1	0
3 yr.	3.3	1	1	0	96	2*b*	2*y*	2
					72	1	2*y*	1
					48	1	1	1
4 yr.	4.5	1	1	1	96	2*b*	2*y*	2
					72	2*b*	2*y*	1
					48	1	1	1

(*Continued on p. 512*)

TABLE V.—STABILITY OF COMMERCIAL OXOPHENARSINE HYDROCHLORIDE (MAPHARSEN) IN AMPULS
(*Continued from p. 511*)

Age	Test Results: Oxophenarsine Hydrochloride Loss, %	Color of Solid	Solution: Color	Solution: Appearance	N. I. H.—Heat Test—70°: Time, Hr.	Color of Solid	Solution: Color	Solution: Appearance
		(*d*) Volatile Content <0.5%, Under Air						
0	0.0	0	0	0	168	2*b*	2*y*	4
					144	2*b*	2*y*	3
					120	1	1	1
					96	1	1	0
6 mo.	0.5	0	0	0	120	1	1	1
					96	1	1	0
1 yr.	1.1	0	0	0	120	1	1	3
					96	1	1	1
2 yr.	2.3	1	1	0	120	2*b*	2*y*	3
					96	1	1	2
					72	1	1	1
3 yr.	3.6	1	1	1	120	4*b*	3*y*	5
					96	3*b*	3*y*	3
					72	2*b*	2*y*	1
					48	1	1	1
4 yr.	5.1	2*b*	1	1	96	5	5	5
					72	2*b*	2*y*	2
					48	2*b*	1	1
		(*e*) Volatile Content <1.0%, Under Air						
0	0.0	0	0	0	120	5	5	5
					72	3*b*	3*b*	3
					48	2*b*	2*y*	1
					24	1	1	1
6 mo.	0.8	0	0	1	72	3*b*	3*b*	3
					48	2*b*	2*y*	2
					24	1	1	1
1 yr.	1.7	1	1	1	48	3*b*	3*y*	2
					24	2*b*	2*y*	1
2 yr.	3.5	2*b*	2*y*	2	48	3*b*	3*y*	3
					24	2*b*	2*y*	2
3 yr.	5.4	3*b*	2*y*	2	48	5	4*y*	5
					24	3*b*	3*y*	3
					16	2*b*	2*y*	3

3. Solution Stability.—*Effect of pH.*—Oxophenarsine hydrochloride was dissolved in oxygen-free water and the *p*H adjusted with hydrochloric acid or sodium hydroxide to the desired range. The solutions were then ampuled under nitrogen and under air. The ampuls were examined at intervals to determine the extent of decomposition. The solutions under nitrogen remained colorless or at most a very pale yellow, while those under air colored and eventually turned black. The rate of darkening was more rapid at *p*H 6 than at *p*H 1.37. Table VI is a composite of the results. The five-year values were obtained from similar solutions prepared by B. F. Tullar. In order to judge the behavior of oxophenarsine hydrochloride in solutions of various *p*H values, the one-month values were plotted as representative of the decomposition rate. They are shown in Fig. 1.

4. Solution Stability.—*Effect of Temperature.*—Solutions of oxophenarsine hydrochloride were prepared as in 3. These solutions were held at various temperatures for one hour and the per cent decomposed determined. The values are graphed in Fig. 2. The study at *p*H 2.7 was performed by J. A. Sultzaberger.

5. Solution Stability in Clinical Use.—Multiple dose packages of commercial oxophenarsine hydrochloride (Mapharsen) were prepared according to directions and diluted to 100 ml. in glass-stoppered graduates. The solutions so prepared were of the same concentration as is used clinically. The samples were subjected to various conditions which might be encountered in use. One graduate was stored in a refrigerator except when samples were withdrawn, the second was kept in diffused light at room temperature, the third was left on the laboratory desk

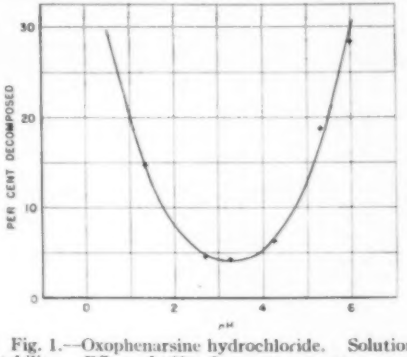

Fig. 1.—Oxophenarsine hydrochloride. Solution stability. Effect of *p*H. Room temperature—one month.

TABLE VI.—OXOPHENARSINE HYDROCHLORIDE STABILITY IN WATER AT ROOM TEMPERATURE

	% Undecomposed								
					pH				
Time	1.37 (N_2)	1.37 (O_2)	2.70 (N_2)	2.70 (O_2)	3.25 (N_2)	4.25 (N_2)	5.30 (N_2)	6.02 (N_2)	6.02 (O_2)
2 hr.	99.9	100.0	100.0	100.0	...	...	...	99.9	99.9
4 hr.	99.8	99.7	99.9	99.9	...	...	...	99.8	99.8
8 hr.	99.6	99.5	99.8	99.8	...	...	...	99.6	99.6
24 hr.	99.3	99.1	99.5	99.5	...	...	...	98.8	98.7
2 days	98.7	98.7	99.3	99.3	...	...	...	97.8	97.6
3 days	...	...	99.1	...	...	...	...	96.3	96.1
1 week	...	...	98.1	98.0	...	...	...	92.1	91.8
2 weeks	92.5	91.9	96.8	96.6	...	...	89.9	84.9	...
1 mo.	85.2	...	95.4	...	95.70	93.7	81.1	71.6	...
2 mo.	74.3	...	91.9	...	91.40	86.6	66.3	50.4	...
3 mo.	62.5	...	88.1	...	87.25	80.8	53.9	36.1	...
4 mo.	53.3	...	84.2	...	83.10	74.8	43.6	25.7	...
5 mo.	45.6	...	80.3	...	79.68	69.9	35.5	18.1	...
6 mo.	38.8	...	76.8	...	75.47	64.7	29.0	12.7	...
7 mo.	33.3	...	...	...	...	...	...	...	...
8 mo.	28.8	...	70.7	...	...	...	...	...	...
1 yr.	15.1	...	59.8	...	56.85	41.8	8.38	trace	...
2 yr.	2.20	...	36.2	...	...	...	...	...	...
5 yr.	trace	...	8.25	...	trace	trace	...	...	...

unstoppered but covered and the fourth was placed on a window sill where it was in direct sunlight. The results are given in Table VII.

DISCUSSION

As was shown in the third paper of this series (1), anhydrous oxophenarsine hydrochloride is far more stable than it is usually considered to be. It undergoes decomposition in the presence of water, and in the presence of oxygen the decompostion becomes very apparent due to the formation of highly colored oxidation products of *o*-aminophenol. The time-temperature study reported in Table I more clearly illustrates the nature of the decomposition. Since it was impractical to prepare an analytically pure sample completely free of water, the nature of the absolutely anhydrous material must be inferred. Considerable information can be gained from Table I, which has been graphed in Fig. 3. It is evident that two reactions are in progress. One reaction rate is greatly affected by heat as evidenced by the sharp upward trend at 50°, 60°, and 70°. The second rate is less influenced by heat and may be nearly absent at lower temperatures. Using the Arrhenius temperature equation, $k = se^{-\Delta H/RT}$, the three-month values for 30°, 40°, and 50° are compatible with a constant temperature increment in reaction rate while the 20° figure is somewhat low. Above 50° the increment is smaller, giving the impression that some limit of reaction was reached before three months. The data at six months are similarly compatible between 20° and 40°. Since it has been demonstrated that the action of water is hydrolytic, an estimation of the extent of reaction due to water can be made. When the water content is 0.05%,

TABLE VII.—SOLUTIONS OF OXOPHENARSINE HYDROCHLORIDE (6 MG./ML.)

Sample	Time, Hr.	Oxophenarsine Hydrochloride, %	Transmission, %	pH
Refrigerator	0	100.0	99.5	5.93
	2	100.0	99.0	5.97
	4	100.0	98.7	6.02
	8	99.7	98.4	6.03
	24	98.9	97.0	6.06
	48	98.9	94.5	6.10
Diffused light	0	100.0	99.5	5.93
	2	99.3	99.0	5.98
	4	98.8	97.0	6.03
	8	98.0	96.4	6.03
	24	97.4	95.8	6.05
	48	97.7	92.0	6.10
Sunlight	0	100.0	99.5	5.93
	2	98.7	97.8	6.26
	4	98.1	94.3	6.33
	8	97.8	89.7	6.37
	24	97.1	66.7	6.40
	48	96.5	41.1	6.43
Open	0	100.0	99.5	5.93
	2	100.0	97.3	6.28
	4	100.3	96.5	6.38
	8	102.4	71.4	6.40
	24	108.2	32.0	6.42
	48	116.6	15.8	6.51

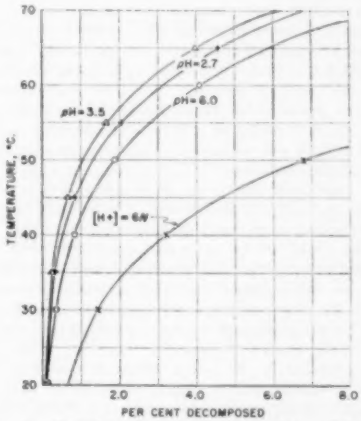

Fig. 2.—Oxophenarsine hydrochloride. Solution stability—one hour. Effect of temperature.

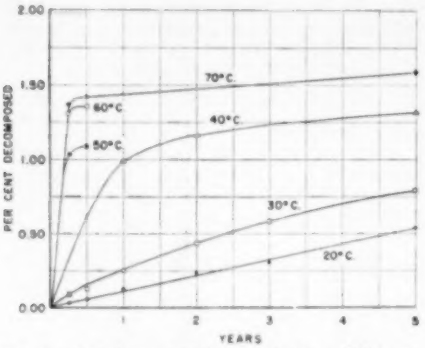

Fig. 3.—Oxophenarsine hydrochloride. Solid stability. Effect of temperature.

this figure is a maximum of either 0.65% or 1.3%, depending on whether the hydrolysis is monomolecular or bimolecular. When the 50°, 60°, and 70° curves are extrapolated back to zero time from five years, the per cent decomposed at zero (extrapolated) fall between 1.3% and 1.35%. This indicates that the initial reaction at these temperatures is a rapid hydrolysis involving two molecules of the arsenical for each molecule of water present, followed by a very slow reaction of dissociation which resulted in a total decomposition of 0.25% in five years. A corresponding extrapolation of the 40° curve gave a zero (extrapolated) value of 1.04% decomposed, indicating that the hydrolytic rate had been appreciably slowed, or altered by the decrease in temperature. The 20° and 30° curves show no differentiation between reactions, both being of such low order as to be scarcely capable of differentiation from analytical error. (It should be appreciated that the best analytical techniques for these determinations are never better than ±0.05% and may vary by ±0.08%.)

Since a correction of the 20° and 30° values for the dissociation reaction would bring the hydrolytic decomposition to less than one-half the 70° value, it is possible that hydrolysis at lower temperatures may result in mole-to-mole reaction rather than the two-to-one figure found at 70°. This hypothesis is substantiated by the data in Tables II, III, and IV in which it is evident that the hydrolytic reaction at 20°, 30°, and 40° involves the decomposition of one mole of arsenical for each mole of water, while at 60 and 70° two moles of arsenical are decomposed for each mole of water present. The total decomposition of samples containing the maximum amount of water allowed by the U. S. P. is about 8% in three years and 10% in five years. These same samples may decompose as much as 20% in six months at 70°.

The time study of commercial mixtures was judged not only on the extent of decomposition but also on other requirements as established by the U. S. P. Some of these requirements not only involve the question of active ingredient but also relate to the clarity of injectable solutions. The samples ampuled in air and in nitrogen with less than 0.05% volatile matter clearly indicate the inherent stability of the product but such low volatile losses are commercially impractical since the product has a hydroscopicity greater than calcium sulfate. When the volatile loss of the injection mixture was less than 0.5% (U. S. P. limit) the arsenical decreased about 5% in four years and the presence or absence of oxygen made little difference in the decomposition rate or total amount of decomposition. There was, an appreciable difference in the appearance of the ampuls under nitrogen as compared with those under air. The ampuls under nitrogen initially passed the heat test at one hundred sixty-eight hours and would probably have gone much longer; the same material under air passed one hundred twenty hours and was questionable at one hundred forty-four hours. While both types of ampuls were still entirely acceptable after four years at room temperature, the nitrogen-filled samples would pass a ninety-six-hour heat test, whereas the air-filled ampuls barely passed a seventy-two hour test and were unsatisfactory after a ninety-six hour heat test. In both instances the material passed the U. S. P. minimum requirements, even after four years.

The group of ampuls having moistures above the U. S. P. limits would barely pass a forty-eight-hour heat test initially and might be considered questionable after three years, although the loss in arsenical activity was only about 5%.

In comparison, there appears to be some correlation between stability, heat test, moisture, and time stability in regard to oxophenarsine hydrochloride mixtures. The relationship is considerably beclouded by a number of factors: the appearance of the mixture bears little or no relationship to the relative potency as far as the arsenical is concerned; the stability to decomposition is affected by water but not in a quantitiative fashion; and the presence or absence of oxygen may or may not affect the appearance of the product. A partial explanation can be effected by considering that hydrolytic decomposition is caused by water, but the decomposition becomes apparent only when oxygen is present. Also, the amount of available water is not the total content, but that portion which is held by the arsenical in equilibrium with the hydrates of sodium carbonate. Further, the decomposition has been shown to follow a different process at 70° than that observed at room temperature.

In general, it can be said that the U. S. P. heat test for oxophenarsine hydrochloride mixtures is more sensitive to deleterious conditions than possibly any other test, including the volatile loss determination. In no instance did a sample which was acceptable by U. S. P. standards change enough in four years to be pharmacologically significant.

Although it has been shown previously (1) that solutions of oxophenarsine hydrochloride decompose, it was of interest to determine the rate of decomposition, particularly with regard to *p*H. The data in Table VI clearly indicate a common decomposition mechanism with a minimum rate around *p*H 3 to 3.5 and increasing when the *p*H is changed in either direction. The plot in Fig. 1 graphically demonstrates this feature. The data for all *p*H values are compatible with a unimolecular reaction mechanism. The minimum at about *p*H 3 is also very suggestive of a particular ion form being more stable than the common ions at other *p*H levels. The temperature-decomposition curves in Fig. 2 confirm the observations at room temperature and indicate the usual Arrhenius relationship between rate constants with increasing temperature.

In an effort to determine the safe period for utilization of solutions made from multiple-dose p ckages of oxophenarsine hydrochloride, the various possible clinical conditions which might be encountered were simulated in the four series of experiments. The sample kept under refrigeration retained 99% of its activity for a week. During this time the *p*H changed slightly from an initial of 5.93 to a final value of 6.10 due to gradual loss of carbon dioxide. During the entire time the color and esthetic appearance were satisfactory. In diffuse light at room temperature the activity was 97-98% after one day, 97% in two days, and 92% in a week. The color change was negligible for the first three days and only moderate at the end of a week and the *p*H change insignificant. In direct sunlight the decrease in activity was the same as in diffuse light but the color change was more rapid and became esthetically undesirable after two days. The unstoppered sample did not lose in activity, but gained, indicating that the decomposition was slower than evaporation. After eight hours the solution had such a dark color as to be undesirable.

SUMMARY

1. Essentially anhydrous oxophenarsine hydrochloride decomposes only slightly over a five-year period at temperatures ranging from 20° to 70°.

2. Oxophenarsine hydrochloride containing moisture to the extent allowed by the U. S. P. shows a characteristic decomposition rate which approaches a limit of one equivalent of arsenical decomposed for each equivalent of water present. Since the amount of water permissible is small, the maximum decomposition is within the tolerance for the product and is too small to detect by pharmacological methods. The temperature increment of the reaction rate corresponds to the Arrhenius theory. At higher temperatures than the product would encounter, the amount of arsenical decomposed for a given moisture content is doubled.

3. Commercial ampuls were stable for four years.

4. Solutions of oxophenarsine hydrochloride decompose by a unimolecular mechanism in a first-order reaction. The minimum decomposition rate is at about *p*H 3.25. Solutions of *p*H 6 are adequately stable for clinical purposes but are not sufficiently stable for use over a long period of time.

5. Solutions made from multiple dose packages are adequately stable for three days when maintained at room temperature or below. Diffused light does no harm but sunlight is deleterious. Such solutions should be kept under a vaporproof stopper to prevent evaporation and subsequent overdosage.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Absinthin
7-Hydroxy-1-H-v-triazolo-(d)-pyrimidine
5,7-Diamino-1-H-v-triazolo-(d)-pyrimidine
7-(α)-12-(α)-Dihydroxyprogesterone
12-(α)-Hydroxyprogesterone
6-Ketoprogesterone
Sulfanylthiourea
Xanthotoxin
Sodium pregnanediol glucuronate
Phloretin
2-Methyl-3-aminopropiophenone
3-Methyl-5-aminopropiophenone
Imperatorin
Hemipic acid
Erythrophleine
O,N-Diacetylsalicylamide
Coniferyl alcohol
2,4-Diamino-6-methylpyrimidine
Muscone
Myristicine
8-Chlorotheophylline
2-Amino-1,3,4-triazole
9-Methyl-2,6,7-trihydroxy-3-fluorone
9-Phenyl-2,6,7-trihydroxy-3-fluorone

Local Irritation Caused by Streptomycin in Animals Compared to Pain Produced in Man*

By S. KUNA and F. T. CUCHIE

Several methods to determine irritation were investigated. The best results were obtained using a procedure essentially the same as that reported by J. K. Finnegan. This test consisted of subcutaneous injections of various concentrations of streptomycin on the outer surface of rabbits' ears. Doses of 0.05 cc. of solutions containing 100,000 u./cc. to 400,000 u./cc. were used. The degree of irritation at the end of forty-eight hours was scored using an arbitrary scale, and the relative irritation of the various samples was evaluated biometrically.

NUMEROUS reports from clinicians using the earlier lots of streptomycin had indicated considerable lot-to-lot variation in the degree of pain following injection. In some cases the intensity of pain after injection was so severe that patients refused further treatment. Since this pain was frequently accompanied by irritation and soreness at the site of injection, we investigated the possibility of using the degree of irritation in animals as a guide to the intensity of pain which might result following injection of the same sample in man.

EXPERIMENTAL

Several methods to determine irritation were investigated. The best results were obtained using a procedure essentially the same as that reported by J. H. Weatherby (1) and later by J. K. Finnegan (2). Our procedure consisted in the subcutaneous injections of various concentrations of streptomycin into the dorsal surface of rabbits' ears. Doses of 0.05 cc. of solutions containing 150,000 units per cc., 200,000 units per cc., and 250,000 units per cc. were used to test each sample. Each dilution was injected in one ear of each of two rabbits. Thus the ears of six rabbits were used to test two lots of streptomycin. The degree of irritation was scored on the extent of erythema, edema, and necrosis, taking into consideration both the area and the depth of the lesion produced. A numerical system of scoring was devised (Table I). The numerical values of the ir-

Fig. 1.—Control—normal saline injected. (Rabbit ear.)

TABLE I.—NUMERICAL SYSTEM FOR EVALUATING THE DEGREE OF IRRITATION

Numerical Value	Standard Method	Degree and Type of Irritation
1	−+	Very slight erythema
2	+−	Slight erythema
3	+− to +	Slight erythema + very slight edema
4	+	Slight erythema + slight edema
5	+ to ++−	Moderate erythema + slight edema
6	++− to ++	Moderate erythema + moderate edema
7	++	Marked erythema + marked edema
8	++ to +++−	Erythema, edema + possible necrosis
9	+++− to +++	Erythema, edema + slight necrosis
10	+++	Erythema, edema + necrosis

* Received April 30, 1949, from the Merck Institute for Therapeutic Research, Rahway, N. J.
Presented to the Scientific Section, A. Ph. A., Jacksonville meeting, April, 1949

Fig. 2.—Sample of streptomycin well tolerated clinically. (Rabbit ear.)

Fig. 3.—Sample of streptomycin causing pain clinically. (Rabbit ear.)

ritation caused by three (standard) dilutions of each sample of streptomycin were recorded at the end of twenty-four and forty-eight hours after injection. The sum of these numerical values was used to represent the degree of irritation caused by the sample.

For this investigation seven lots of streptomycin which had caused pain on injection in man were compared with samples that were well tolerated. Those samples which were painful on injection also caused a more marked irritation in the rabbits' ears. The numerical score of these samples fell between 48 and 100, while that of the clinically well-tolerated samples was between 17 and 26 (Table II).

TABLE II.—RELATIONSHIP BETWEEN CLINICAL FINDINGS AND DEGREE OF IRRITATION IN RABBITS

Sample No.	Salt	Reaction at Site of Injection (Man)	Degree of Irritation (Rabbit Ear)
Lot 598	HCl	Pain	76
Lot 680	HCl	No pain	22
Lot 9694	$CaCl_2$	No pain	22
Lot 613	HCl	Pain	92
Lot 558	HCl	Pain	54
Lot 533	HCl	Pain	54
Lot 661	HCl	No pain	25
Lot 563	HCl	Pain	48
Lot 682	HCl	No pain	26
Lot 6981	$CaCl_2$	No pain	22
Lot 596	HCl	Pain	90
Lot 560	HCl	Pain	58
Lot 1334	$CaCl_2$	No pain	23

One sample, Lot 613, which was clinically the most painful on injection and which gave the highest numerical score in the rabbit test, was purified by the chemists of Merck & Co., Inc., from the original 50% purity to a purity of 96%. The purified preparation was well tolerated on parenteral injection in man and was also definitely less irritating in the rabbit ear test.

To illustrate the various degrees of irritation that were produced by the various preparations tested, pictures of the irritated areas were taken by transillumination (Figs. 1, 2, and 3).

CONCLUSION

The degree of irritation caused by subcutaneous injection of streptomycin into the outer surface of rabbits' ears shows a reasonable correlation with the intensity of pain produced clinically. This procedure is proposed as a satisfactory criterion for judging the intensity of pain that a given lot of streptomycin might be expected to cause on administration to patients.

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An Accurate and Economical Method for the Biological Assay of Aconite Tincture*

By MARTIN BARR† and JOHN W. NELSON‡

A method is described for the quantitative assay of aconite tincture. The principle employed involves the administration of intravenous injections of the tincture in mice. A comparison of LD_{50} doses of Reference Standard Aconitine Solution with diluted tinctures is employed for the assay. The standard error of a series of assays was found to be less than two per cent.

AN ACCURATE, SIMPLE, quick, and inexpensive method has been devised for the quantitative assay of aconite tincture, using intravenous injections in mice, in which the comparison of LD_{50} doses of a Reference Standard Aconitine solution and of diluted tinctures is used as the basis for assay. The standard error of each series of assays was found to be less than 2 per cent.

Aconitum napellus has been official in every U. S. Pharmacopœia from I to XI, inclusive, and in National Formulary VII and VIII. However, there has never been a completely satisfactory method for its standardization. It is widely recognized that aconitine is one of the most potent alkaloids known, and, therefore, any assay procedure which gives a product of variable toxicity is dangerous. It has largely been because of this fact that aconite has fallen into disuse.

Dunstan (1) and Freund (2) showed that the principal active alkaloids of aconite were aconitine, benzoylaconine, and aconine. Pharmacological tests indicate that aconitine is 500 times as toxic as benzoylaconine and 5000 times as toxic as aconine. It also has been quite generally conceded that the activity of aconite is due to aconitine, which is the chief alkaloid, both qualitatively and quantitatively.

Aconite tincture was decided on as the preparation of aconite to be studied, since it is the most commonly used of aconite preparations in medicine today.

A chemical method of assay is most desirable when such a method is possible; however, since Dohme (3) reported that there is no satisfactory method of separating the active constituents of aconite, it appears that a chemical method of assay does not lend itself to this drug. Furthermore, Rudolph and Cole (4), Robinson (5), Ford, *et al.* (6), Haskell (7, 8), and Haskell and Zirkle (9) and others found that a chemical assay based on total alkaloidal content is unreliable. Therefore, it appears that a biological assay must be resorted to.

In 1921 Dohme (10) developed a M. L. D method for determining aconite potency, making use of guinea pigs as the test animals. This method was improved in 1923 by Swanson and Walters (11) and in 1924 by Swanson (12). Rowe (13) advocated the use of white mice in place of guinea pigs, stressing the economy. Hoppe and Mollett (14) introduced a suggested assay for aconite tincture, in which they used the determination of the lethal effect of reference aconitine and of a tincture of aconite as their basis for assay.

The present official guinea-pig method of assay for aconite tincture has been highly recommended because of its constancy. However, there are several outstanding objections to this method: the lack of sensitivity and the time factor and expense involved in the assay procedure.

By observing the present N. F. VIII (15) allowance of ±20 per cent, two official tinctures of aconite may vary in strength by 40 per cent in terms of the official standard, which is Reference Standard Aconitine. Considering that the active constituent of aconite tincture, aconitine, has an average dose of 1 to 400 grain and that its therapeutic ratio is very small, the variable toxicity and lack of safety introduced by this variation become significant.

The six-hour end point of the N. F. VIII assay for aconite tincture has been considered a drawback to the method. It increases labor and observation time, and together with the cost of the number of guinea pigs required for the procedure, it raises the expense of the assay.

In this work an attempt was made to develop an assay procedure for aconite tincture which will give a more precise measure of the potency of the tincture than that obtained by the present official N. F. VIII method; and also, a procedure which will consume less time and be less expensive

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than the present official method; in brief, to develop a procedure possessing the practical attributes of a good biological assay: simplicity, rapidity, economy, a definite and unmistakable end point, and a reasonable degree of accuracy.

EXPERIMENTAL

An "80% known aconitine solution," two recently standardized official tinctures obtained from reliable pharmaceutical concerns, and a tincture prepared extemporaneously were assayed several times using the LD_{50} for N. F. Reference Standard Aconitine as the basis for standardization. The end point selected in this work was based on the determination of the LD_{50} dose; i.e., the doses of the reference aconitine and the preparation being assayed which killed 50% of the animals injected, at the end of a ninety-minute observation period.

The mice used in the assays were of a uniform strain and sex (Swiss males). They were selected from a colony kept under identical conditions and fed on a continuous diet of Purina Dog Pellets and water. They were housed in metal cages at room temperature. Mice were not used unless they had been previously observed for one week. Because of the acknowledged rapid excretion of aconitine, some of the mice were used more than once, but they were given at least seven days to recover from any possible aconitine effects before re-use. Mice weighing from 16.0 Gm. to 24.0 Gm. should be used for optimum results.

The solution of Reference Standard Aconitine was prepared according to directions in the official method for assay of aconite tincture in the Eighth Edition of the National Formulary. Dilutions were prepared with distilled water (pH 3 ± 0.2) in such a manner that 1 cc. of solution of reference aconitine contained 0.01 mg. of N. F. Reference Standard Aconitine. In this manner, the volume of the injected doses varied from the extremes of 0.11 cc. to 0.56 cc., with the average volume of injection being 0.26 cc. to 0.44 cc. Injections were made intravenously in the tail veins of the mice, at a slow uniform rate. A 0.5-ml. or a 1.0-ml. tuberculin syringe was used for all injections, whichever was more convenient for the individual injections. The needles used were of a 25-gauge and $^5/_8$-inch length.

All solutions and dilutions were freshly prepared just previous to the time of use in order to insure against possible loss of lethal activity due to hydrolysis of the preparations.

Dilutions of the "80% known aconitine solution"[1] and the tinctures to be assayed were prepared in the above-described manner to give the same volume of dosage.

A graduated series of doses of the Reference Standard Aconitine dilution (1 cc. = 0.01 mg. R. S. Aconitine) and the dilution of the "80% known aconitine solution" (1 cc. = 0.008 mg. R. S. Aconitine) were injected intravenously and the LD_{50} determined at the end of a ninety-minute observation period. The strength of the preparation being assayed was then determined by direct proportion between the concentration of the "80% known aconitine solution" and the amount of reference standard that resulted in an LD_{50} in both cases.

In Table I the results of four assays of the "80% known aconitine solution" are given. The results of the assays are well within the limit of the "80% aconitine" content, and indicate that the method of assay for aconitine content is indicative of the actual aconitine value.

TABLE I.—PERCENTAGE STRENGTH OF A "80% KNOWN ACONITINE SOLUTION" ASSAYED BY MEANS OF THE MOUSE METHOD

Assay No.	LD_{50} of R. S. Aconitine Solution	No. of Mice	LD_{50} of "80% Known Aconitine Solution"[a]	No. of Mice	Calculated % Strength of "80% Known Aconitine Solution"
1	0.0001542	42	0.0001800	42	85.67
2	0.0001377	42	0.0001675	42	82.21
3	0.0001550	42	0.0001969	42	78.72
4	0.0001560	42	0.0002013	42	77.50

[a] Mg. aconitine per Gm. mouse weight.

The aconite tinctures were arbitrarily regarded as being equivalent in aconitine potency to the reference standard solution (1 cc. = 0.15 mg. aconitine). Dilutions of the tinctures were prepared similarly to the dilutions of the reference standard solution so that each 1 cc. of dilutions of the tinctures was equivalent in aconitine potency to each 1 cc. of dilutions of the reference solution (1 cc. = 0.01 mg. aconitine).

A graduated series of doses of the reference standard dilution and the diluted tinctures were injected intravenously and the LD_{50} determined after a ninety-minute observation period. The strength of the tincture being assayed was then determined by direct proportion between the concentration of the tincture and the amount of reference aconitine that resulted in a LD_{50} in both cases. The present official requirement is that 1 cc. of aconite tincture be equivalent in action to 0.15 mg. of N. F. Reference Standard Aconitine and this is taken to represent 100% of the required potency.

In Table II, the results of the assays of three tinctures are given. The two recently standardized

TABLE II.—PERCENTAGE STRENGTHS OF THREE ACONITE TINCTURES ASSAYED BY MEANS OF THE MOUSE METHOD

Assay No.	LD_{50} of R. S. Aconitine Solution[a]	No. of Mice	LD_{50} of Aconite Tincture[a]	No. of Mice	Calculated % Strength of Aconite Tincture
			Tincture A		
1	0.0001681	42	0.0001426	42	117.8
2	0.0001569	42	0.0001391	42	112.8
3	0.0001570	42	0.0001390	42	112.9
				Av. % Tincture A	114.5
			Tincture B		
1	0.0001530	42	0.0001467	42	104.3
2	0.0001500	42	0.0001477	42	101.6
3	0.0001567	42	0.0001476	42	106.2
				Av. % Tincture B	104.0
			Tincture C		
1	0.0001453	42	0.0001359	42	106.9
2	0.0001533	42	0.0001383	42	110.8
3	0.0001513	42	0.0001429	42	105.9
				Av. % Tincture C	107.9

[a] Mg. aconitine per Gm. mouse weight.

[1] An "80% known aconitine solution" represents a solution containing 80% of the aconitine content of the official N. F. Reference Standard Aconitine Solution (i.e., 1 cc. contains 0.12 mg. of Reference Standard Aconitine).

official tinctures obtained from manufacturing concern are designated as Tinctures A and B. Tincture C represents the aconite tincture prepared in this laboratory prior to assay. The widest variation is about 5% which is well within the N. F. VIII requirements of ±20%.

The "80% known aconitine solution" and the three tinctures of aconite (Tinctures A, B, and C) were assayed according to the directions of the official guinea-pig assay for aconite tincture, N. F. VIII. The tinctures were found to meet official requirements; i.e. they assayed 100% in terms of the N. F. Reference Standard.

A statistical interpretation of data as shown in Table III, indicates that the intravenous mouse method of assay of aconite tincture is reasonably accurate. The standard error in a series of assays on the same tincture or upon an "80% known aconitine solution" was found to be less than 2% (1.34% to 1.84%).

utes; and 97% of the animals, which were eventually to die, did so within sixty minutes. However, because of a few "borderline cases," it was decided to use a ninety-minute observation period to avoid any possible errors in denoting deaths. Animals living ninety minutes after injection survived indefinitely. In this work, dealing with well over 1200 mice, only four died after the ninety-minute designated end point, and these deaths were attributed to factors other than aconitine toxicity.

DISCUSSION

The tinctures of aconite, which were assayed according to the present N. F. VIII guinea pig method of assay, were found to meet official specifications. However, this indicates only an approximate idea of the potency of the tinctures, as the

TABLE III.—STATISTICAL INTERPRETATION DATA[a]

	Preparation Bioassayed			
	"80% Known Aconitine Solution"	Aconite Tincture, % Strength		
		Tincture A	Tincture B	Tincture C
$M = \frac{\Sigma X}{n}$	81.025	114.50	104.03	107.87
$M.D. = \frac{\Sigma d}{n}$	2.915	2.20	1.62	1.96
$S.D. = \sqrt{\frac{\Sigma d^2}{n}}$	3.68	2.86	2.31	2.59
$S.E. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$	1.84	1.65	1.34	1.49

[a] These statistics were checked by the Statistics Laboratory of The Ohio State University.

The average LD_{50} dose of reference aconitine was found to be 0.000153 mg./Gm. body weight of mouse. In this work, however, it was noted that the LD_{50} doses vary from day to day to a certain extent (as may be seen in Table II); therefore, all LD_{50} determinations of the reference standard aconitine solutions and of the tinctures must be made during the same experimental period in order to simulate identical conditions as much as possible and to reduce any existing source of error.

For the determination of the LD_{50} doses, the double integration method of Behren (16) was employed. This method enables one to obtain results in terms of a much larger number of animals than originally employed, and, therefore, results are indeed more accurate. In Table IV is shown a typical example for the determination of the potency of a tincture by comparing the LD_{50} dose of a reference standard solution of aconitine with the LD_{50} dose of a tincture of aconite. Note that although only 84 animals were originally employed, the results are expressed in terms of 230 animals.

The ninety-minute observation period was established after study of the time-death relationships. Dead animals were counted at intervals of fifteen minutes. Seventy-five per cent of the animals, destined for death, died within fifteen to thirty minNational Formulary does not require the exact potency of the tinctures to be determined.

Using the described mouse method of assay in assaying the same tinctures of aconite, it is possible to assign a more definite potency to each tincture, whereby the same tincture does not vary by more than 5% (Table II). The standard error of each series of assays was found to be less than 2% (1.49–1.65%).

The low margin of error produced in the mouse method of assay is brought about by several factors: (*a*) the double integration method of statistical interpretation of Behren, making it possible to interpret results in terms of approximately three times as many animals as actually used in the assay; (*b*) the definite and quick toxicity of aconitine, making possible a short observation period which reduces the possibility of producing variabilities in experimental conditions; (*c*) the intravenous route of injection, which eliminates possible loss of drug as sometimes occurs by other routes of administration, as well as making possible a shorter observation period due to the fact that the drug need not be absorbed.

In addition to the above advantages, the mouse method has economical advantages over the N. F. VIII method. Mice are less expensive laboratory

TABLE IV.—ASSAY OF AN ACONITE TINCTURE USING THE MOUSE METHOD OF ASSAY AND BEHREN'S METHOD FOR STATISTICAL DETERMINATION OF LD_{50} DOSES

Dose in Mg./Gm. Body Wt.	Experimental Data			Integrated Data		
	Alive	Dead	Mortality, %	Alive	Dead	Mortality, %
	(*a*) Integration of Reference Aconitine LD_{50} Determination					
0.000070	6	0	....	23	0	....
0.000100	5	1	16.67	17	1	5.56
0.000130	5	1	16.67	12	2	14.29
0.000150	3	3	50.00	7	5	41.67
0.000160	2	4	66.67	4	9	69.23
0.000190	2	4	66.67	2	13	86.67
0.000220	0	6	100.00	0	19	100.00
Totals	23	19		65	49	
LD_{50} dose = 0.0001530 mg./Gm. body weight of mouse.						
	(*b*) Integration of Aconite Tincture B LD_{50} Determination					
0.000070	6	0	....	20	0	....
0.000100	5	1	16.67	14	1	6.67
0.000130	3	3	50.00	9	4	30.77
0.000150	3	3	50.00	6	7	53.85
0.000160	1	5	83.37	3	12	80.00
0.000190	2	4	66.67	2	16	88.89
0.000220	0	6	100.00	0	22	100.00
Totals	20	22		54	62	

LD_{50} = 0.0001467 Mg./Gm. body weight of mouse.

LD_{50} Reference Aconitine × 100 = % Strength Aconite Tincture in Terms of Reference Standard Aconitine
LD_{50} Aconite Tincture B, Assay No. 1

$$\frac{0.0001530 \text{ Mg./Gm. Reference Aconitine} \times 100}{0.0001467 \text{ Mg./Gm. Aconite Tincture B, Assay No. 1}} = 104.3\% \text{ Strength of Aconite Tincture in Terms of Reference Standard Aconitine}$$

Preparation—Aconite Tincture B. Worker—M. B.
% Strength—104.3% Date—10-10-48.

animals than guinea pigs and are easier to house. Also, the ninety-minute observation period is a definite time saver over the present guinea-pig assay six-hour end point. A complete assay of aconite tincture may be completed in a period of three to four hours by experienced workers, whereas the guinea-pig assay requires well over six hours for completion.

CONCLUSIONS

1. An assay method of relatively high reproducibility has been developed for the biological assay of aconite tincture, using intravenous injections in mice, in which the comparison of LD_{50} doses of a reference standard aconitine solution and of diluted tinctures is used as the basis for assay. The standard error of each series of assays was found to be less than 2 per cent.

2. The use of mice affords a simple and economical means of biologically assaying a tincture of aconite. The use of a ninety-minute observation period also results in the reduction of the time required to perform a complete assay as compared to the present official N. F. VIII method.

3. The described method offers a biological procedure for assay of tinctures of aconite which is less expensive, simpler, quicker, and more accurate than the present guinea-pig method.

4. The described method should be applicable to other preparations of aconite.

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Sabadilla Alkaloids. I. Extraction of Total Alkaloids*

By CHESTER E. POETSCH† and LLOYD M. PARKS

A method is described by which the total sabadilla alkaloids were extracted from the seed and by which a partial separation of the crude alkaloidal mixture into four crude fractions was accomplished. Extraction of the seed with Skelly B removed the oil almost completely, in amounts up to 20 per cent, together with alkaloidal Fraction A, which was shaken out of the Skelly extract with acid. Extraction of the defatted seed with alcohol removed the remaining alkaloids. Concentration of this extract to a syrup and pouring into cold water yielded a precipitate of Fraction B which contained a considerable amount of alkaloid along with nonalkaloidal matter, probably resin. The filtrate from Fraction B, upon being made alkaline with ammonia, deposited Fraction C, which was removed by filtration. Chloroform extraction of the filtrate from C yielded Fraction D. From 15.5 Kg. of seed the following approximate amounts of the above fractions were obtained: A, 152 Gm.; B, 800 Gm.; C, 220 Gm.; D, 168 Gm.

INTEREST has been revived recently in the sabadilla alkaloids because of their use in insecticides and because of advancements in the chemistry and pharmacology of related alkaloids. Little is known about the individual alkaloids which make up the alkaloidal mixture obtained from sabadilla and commonly known as "veratrine." The present work was undertaken in an attempt to isolate in a pure state the various alkaloids which make up this mixture and thereby make possible a comprehensive chemical and pharmacological study of each component.

Sabadilla belongs to the *Liliaceae* family. The alkaloids are obtained from the dried ripe seeds of *Schoenocaulon officinale* A. Gray. In the earlier literature sabadilla is referred to as Veratrum Sabadilla or *Asagraea officinalis* Lindley. A review of the pharmacognosy of sabadilla is given by Allen, *et al.* (1).

Sabadilla seed usually yields from 3 to 6 per cent total alkaloids and from 15 to 20 per cent fixed oil. The total alkaloids constitute a mixture which has been reported to contain cevadine (crystallized veratrine), veratridine (amorphous veratrine), cevadilline (sabadilline), sabadine, and cevine (sabadinine). Undoubtedly other unidentified alkaloids occur in this mixture.

Of the five components reported to make up the mixture only cevadine, veratridine, and cevine have been studied to any extent. Some doubt exists as to the purity of veratridine, and the existence of cevadilline and sabadine has not been confirmed. A critical discussion of the early work is given by Wright and Luff (2), while the work up to 1939 is discussed by Henry (3).

The alkaloid now known as cevadine was first isolated by G. Merck (4) in 1855. He assigned the name veratrine to it. The same alkaloid was subsequently obtained by Schmidt and Koppen (5) and by Wright and Luff (2). The latter, in order to distinguish it from the mixture of amorphous bases sold in commerce as "veratrine," introduced the name cevadine which has been generally accepted. They also assigned to it the correct molecular formula, $C_{32}H_{49}O_9N$. Upon hydrolysis cevadine yields the alkanolamine cevine, $C_{27}H_{43}O_8N$, and tiglic acid, $C_5H_8O_2$; it is the tiglic acid ester of cevine (2, 6, 7).

Veratridine, $C_{36}H_{51}O_{11}N$, was the name first ascribed to this alkaloid by Bossetti in 1883 (8). It apparently is identical with Schmidt and Koppen's water-soluble amorphous veratrine (9) obtained by both Wright and Luff (2) and Merck (4). The name amorphous veratrine has fallen into disuse because of the confusion with commercial "veratrine" which is the amorphous total alkaloids from sabadilla seed. Upon hydrolysis veratridine yields cevine, $C_{27}H_{43}O_8N$, and veratric acid, $C_9H_{10}O_4$; it is the veratric acid ester of cevine.

Considerable confusion exists in the literature concerning the alkaloid or alkaloids cevadilline, sabadilline, and sabatrine. Cevadilline, $C_{34}H_{53}O_9N$, was reported by Wright and Luff in 1878. They reported that upon hydrolysis it yields a base which they called cevilline, $C_{29}H_{47}O_7N$, and cevadic acid, $C_5H_8O_2$, probably identical with tiglic acid. Further study is necessary to establish definitely the physical and chemical characteristics of this alkaloid. The sabatrine of

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Weigelin was regarded by Wright and Luff as a mixture of alteration products of other bases. Sabadine was reported by E. Merck (10) in 1890 who ascribed to it the formula $C_{29}H_{51}O_8N$. Apparently, it is an alkamine of the cevine type.

Cevine, $C_{27}H_{43}O_8N$, was first prepared by Wright and Luff (2) in an amorphous form upon hydrolysis of cevadine. Freund and Schwarz (11) were able to obtain it in a crystalline state also from the hydrolysis of cevadine. Hess and Mohr (12) showed cevine and Merck's sabadinine (10) to be identical. Since cevine is the common hydrolysis product of the two main ester alkaloids of sabadilla, cevadine and veratridine, considerable work has been done on its structure. The most extensive work on this subject has been conducted by Jacobs, Craig, and co-workers at the Rockefeller Institute for Medical Research, who carried out experiments not only on cevine but also on some of the veratrum alkamines in an attempt to correlate their observations. The exact structure of cevine is still unknown but basic structures for it have been proposed (13, 14).

In the present work, after various methods of extraction were tested on small scale, there was used a large-scale method to extract the total alkaloids from sabadilla and to separate them into crude fractions, based somewhat on differences in solubility (Fig. 1).

EXPERIMENTAL

Fraction A.—Fifteen and one-half kilograms of finely ground sabadilla seed was extracted with petroleum ether (Skelly B) in a Soxhlet-type extractor. At the end of forty-five hours the extract returning to the pot still left an oily residue upon evaporation but gave no test with Mayer's reagent. When the extraction was stopped, after eighty-eight hours, the solvent was still removing small amounts of oil. The yellow Skelly B extract (14 L.) was filtered while hot and then extracted in separatory funnels with 10% H_2SO_4 until the acid washings no longer gave a test with Mayer's reagent (total washings, 25 L.). Removal of the Skelly B *in vacuo* left a residue of 3350 cc. of oil.

The amber-colored acid washings were washed with Skelly B, filtered, and the filtrate made alkaline with strong ammonia water. The turbid mixture thus obtained was extracted in separatory funnels with chloroform until the chloroform washings no longer gave a test with Mayer's reagent. The pale yellow chloroform extract (23 L.) was then dried over anhydrous Na_2SO_4, filtered, and distilled to dryness *in vacuo* to yield 152 Gm. of pale tan-colored alkaloid Fraction A (see Fig. 1).

Fraction B.—The dried marc remaining from the Skelly B extraction was next extracted continuously with alcohol, with a fresh charge of solvent at the end of seven days. When the extraction was stopped, after sixteen days, the extract returning to the pot yielded upon evaporation a brown residue which did not give a test with Mayer's reagent.

The alcoholic extract was concentrated *in vacuo* to a brown syrup (3 L.). This was poured while hot into 12 L. of water with vigorous stirring which was continued for six hours. The brown precipitate that formed was filtered by suction and washed repeatedly with cold water. Since it became gummy at this stage it was kneaded with Skelly B and then washed repeatedly with Skelly B to remove additional oil. This left 800 Gm. of a tan- to light-brown-colored alkaloid Fraction B which contained, in addition, nonalkaloidal material (probably resin).

Fraction C.—The filtrate and washings remaining from Fraction B were extracted with Skelly B to remove any oil and Skelly B-soluble alkaloids and then were made alkaline with strong ammonia water. The mustard-colored precipitate which formed was filtered by suction and washed 3 times with cold water. After drying on the suction filter and then in a vacuum desiccator for several days the material was still very wet. It was dissolved in 2 L. of 7% H_2SO_4, the dark brown solution shaken out thoroughly with chloroform, and the acid layer then made alkaline with 15% NH_4OH. The resulting mixture, containing a precipitate, was extracted with chloroform, the chloroform extract filtered, dried, and distilled to dryness *in vacuo* to yield 206 Gm. of tan-colored alkaloid Fraction C. A small amount (7.2 Gm.) of chloroform-insoluble solid was removed from the alkaline layer by suction filtration.

Fraction D.—The dark amber alkaline filtrate remaining from above, after filtration to remove Fraction C, was shaken out thoroughly with chloroform until the washings no longer gave a test with Mayer's reagent. The chloroform layer thus obtained was dried and distilled to dryness *in vacuo* to yield 168 Gm. of tan-colored alkaloid Fraction D.

The Skelly B washings which had accumulated in the separation of Fractions B, C, and D were shaken out with acid, the acid layer washed free from color with chloroform and made alkaline with 15% NH_4OH. The resulting mixture was shaken out with chloroform, the chloroform extract dried and distilled to dryness *in vacuo* to yield an additional 16.8 Gm. of pale tan-colored alkaloid material.

pH Separation of Alkaloid Mixture.—In some preliminary experiments attempts were made to effect a separation of the total alkaloids of sabadilla based upon differences in basicity of the individual alkaloids. In one experiment 50 Gm. of alkaloids, obtained by acetone extraction and suitable purification was dissolved in dilute acetic acid, the *p*H of the solution raised by small increments by the addition of dilute ammonia, and the resulting precipitates removed by shaking out with ether. In this way the total alkaloids were separated into several different fractions. In such a separation the weakest base should be precipitated first while the strongest should be precipitated last. In view of the method used, however, the partition coefficients of the individual alkaloids were complicating factors.

Theoretically, the degree of separation possible by this method is governed, in the last analysis, not only by the difference in the basicities of the components but also by the solubility of the undissociated bases in water. It can be shown that at a given *p*H the solubilities of these alkaloids are approximately proportional to the products of their dissociation

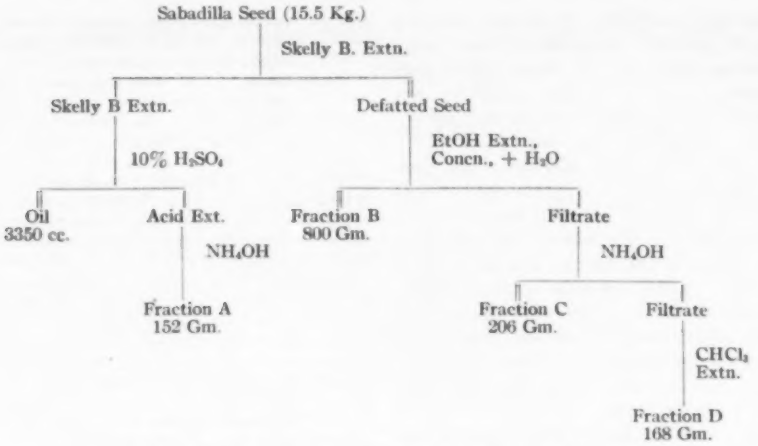

Fig. 1.—Flow sheet for extraction of total sabadilla alkaloids.

constants and their solubility as free bases in water.

The fractions obtained by this method of separation, when tested on the large milkweed bug, *Oncopeltus fasciatus* (Dallas), possessed marked differences in relative toxicity.[1] The fractions which precipitated at the lower *p*H ranges (7.7 to 8.3) were much more highly toxic while those which precipitated at the higher *p*H ranges (8.5 to 9.5) were much less toxic than the original mixture.

Although this and other experiments indicated that the method offered possibilities, further examination of the various fractions showed that there was overlapping of individual components in each fraction and that cevadine and veratridine could be obtained in more or less amounts from several of the fractions. Hence, a sharp separation of alkaloids had not been accomplished. Furthermore, there was indication that partial hydrolysis of some of the ester alkaloids may have occurred during the treatment.

concentrated extract into water yielded a brown material which contained a considerable amount of alkaloids plus other nonalkaloidal matter, probably resin; this was designated as alkaloid Fraction B.

The aqueous filtrate from Fraction B, upon being made alkaline, deposited a large amount of precipitate which was designated as alkaloid Fraction C. Extraction with chloroform of the alkaline filtrate from Fraction C yielded an additional amount of alkaloid designated as Fraction D.

Further examination of these four alkaloid fractions for their individual components is in progress.

SUMMARY

Extraction of sabadilla seed with Skelly B removed almost completely the oil, in amounts of more than 20 per cent, together with Skelly B-soluble and oil-soluble alkaloids, designated as alkaloid Fraction A.

Extraction of the thus defatted seed with alcohol removed the remaining alkaloids plus a trace of oil and some resinous matter. Pouring this

[1] We are indebted to Mr. P. V. Stone, formerly of the Dept. of Economic Entomology, University of Wisconsin, for conducting the toxicity tests.

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Sabadilla Alkaloids. II. Alkaloidal Components of the Petroleum Ether Extract*

By CHESTER E. POETSCH,† TAKERU HIGUCHI, and LLOYD M. PARKS

Crude alkaloid Fraction A (see paper I) was subjected to further separation procedures by fractional solution in Skelly B, followed by fractional crystallizations and recrystallizations. It was not possible to realize a clear-cut separation of pure alkaloids by these procedures. Pure crystalline cevadine, a nearly pure veratridine, and mixed crystals of cevadine and veratridine were obtained. A preliminary separation and purification of some of the fractions was accomplished by means of selective adsorption and elution from an aluminum oxide column. Separations of the various fractions were followed by use of spectophotometric analysis, using the Beckman quartz spectrophotometer. On the basis of absorption spectra, several of the fractions, obtained in small amounts, appeared to be different from any previously reported alkaloids of sabadilla.

A METHOD of extraction and partial separation of the total alkaloids of sabadilla seed into four crude fractions was described in the first report (1). In this report there is described the further fractionation of alkaloid Fraction A, obtained from the petroleum ether extract of the seed, into its alkaoloidal components.

Repeated fractional recrystallizations from petroleum ether yielded fairly pure cevadine which was further purified by recrystallization from alcohol. The veratridine obtained by this procedure could be purified only with difficulty; it was obtained in a high state of purity by conversion to its crystalline sulfate and repeated recrystallization of that salt. No conclusive evidence was obtained for the presence, in significant quantity in Fraction A, of alkaloids other than cevadine and veratridine.

Difficulty in isolating pure alkaloids from the mixture, due to the lack of a good means for following fractionation and isolation procedures, was overcome to a great extent by the use of spectrophotometric methods. The pure alkaloids were found to possess characteristic absorption spectra in the ultraviolet region. This property was found to be very useful in following the purification work.

EXPERIMENTAL

Fractional Recrystallization of Fraction A (see Fig. 1).—One hundred and forty-five grams of pale tan-colored alkaloid Fraction A (1), $[\alpha]_D^{25} = +9.7°$,[1] was extracted with petroleum ether (Skelly B) in a Soxhlet extractor for one week. At the end of this time the extract returning to the pot gave no test with Mayer's reagent, but considerable alkaloidal material remained unextracted (A_1, 28.5 Gm., $[\alpha]_D^{25} = 0°$). The Skelly B extract was distilled to dryness *in vacuo* and the residue obtained in this way was subjected to another extraction with Skelly B for forty-eight hours. At the end of this time insoluble material had deposited in the pot and the extract returning to the pot still gave a test with Mayer's reagent. The insoluble gray mixture remaining unextracted was dried (A_1, 44.5 Gm., $[\alpha]_D^{25} = +6.7°$).

The Skelly B extract (2600 cc.) was filtered while hot through a heated funnel; the insoluble deposit was dissolved in ether-alcohol and this solution distilled to dryness *in vacuo* (A^1-P, 6.3 Gm., $[\alpha]_D^{25} = +8.7°$). The white precipitate, which separated from the Skelly B filtrate upon cooling to room temperature, was filtered, washed, and dried (A_2^1, 14.3 Gm., $[\alpha]_D^{25} = +8.1°$). Cooling the filtrate to 0° with stirring gave an additional white deposit (A_3^1, 6.7 Gm., $[\alpha]_D^{25} = +10.5°$). Warming the filtrate on the steam bath caused the separation of more white material; after again cooling to 0° this was collected (A_4^1, 23.4 Gm., $[\alpha]_D^{25} = +11.4°$, m. p. 204–206°, with previous softening).[2]

The filtrate remaining from the above deposits was concentrated to 300 cc. and cooled to collect another white precipitate (A_5^1, 0.6 Gm., $[\alpha]_D^{25} = +11.1°$ m. p. 202–203.5°, with previous softening). A final concentration to dryness gave only a small amount of impure gummy material (A_6^1, 0.2 Gm.).

Recrystallization of A_4^1; Isolation of Cevadine.—Twenty and nine-tenths grams of Fraction A_4^1 were dissolved by refluxing in 100 cc. of alcohol, the solution filtered, allowed to cool, and seeded with a particle of the solid. After several days' standing at 0° the white crystalline cevadine which had formed was collected, washed, and dried, 16.6 Gm., m. p. 207.5–208.5°, with previous darkening. Working up of the mother liquids yielded a second crop of 3.5 Gm., m. p. 205.5–206.5°, and subsequent crops of colored, impure fractions.

Recrystallization of the cevadine from absolute alcohol yielded various crystal crops, m. p. 208.5–

* Received April 30, 1949, from the School of Pharmacy, University of Wisconsin, Madison.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

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[1] Alcohol was used as the solvent for all optical rotations.

[2] All melting points obtained by Bureau of Standards calibrated Anschutz thermometers.

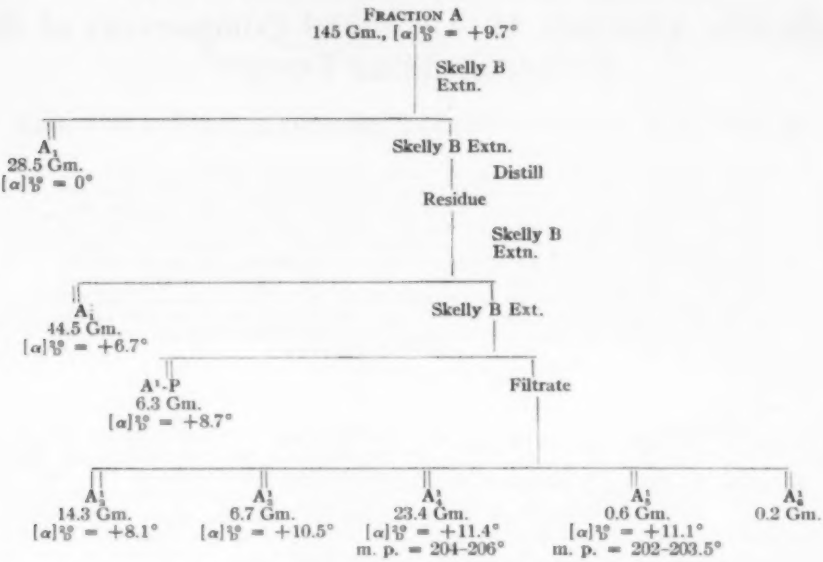

Fig. 1.—Flow sheet of fractional recrystallization of Fraction A.

209.5°, with previous softening, $[\alpha]_D^{30} = +12.8°$ (C = 3.2% in alcohol); and m. p. 209.5–210.5°, $[\alpha]_D^{30} = +12.7°$. These melting points are higher than those reported in the literature for cevadine but its optical rotation and other physical characteristics were the same as those reported. Macbeth and Robinson (3) report $[\alpha]_D^{17} = +12.5°$ (alcohol) and Freund and Schwarz (4) report m. p. of 205°. Further recrystallizations from alcohol and washing with ether raised the m. p. of the highest melting sample to 211–212° (dec.), with softening at 210.5°.

Cevadine Dilituarte.—Two-tenths gram of the purest sample of cevadine and 0.08 Gm. of dilituric acid were dissolved in 10 cc. of boiling water. After standing at room temperature for several days, followed by cooling at 5° for several weeks, the crystalline dilituarte was collected by suction and recrystallized from boiling water, m. p. 222° (dec.), with previous darkening. Ikawa, *et al.*, (2), reported the m. p. of their cevadine dilituarte as 220°.

Recrystallization of A_2^1 and A_3^1.—Because of the apparent success in fractionating crude Fraction A by means of fractional crystallization from Skelly B, this procedure was applied to fractions A_2^1 and A_3^1. Twelve and one-half grams of A_2^1, $[\alpha]_D^{30} = +8.1°$, was refluxed with 1600 cc. of Skelly B, the mixture filtered while hot, and the insoluble residue washed with the hot solvent. Dissolution of the insoluble residue in ether, followed by distillation *in vacuo*, yielded 3.1 Gm., $[\alpha]_D^{30} = +5.3$, m. p. indefinite. Fractional crystallization of the filtrate yielded the following crops: 3.5 Gm., $[\alpha]_D^{30} = +6.9°$, m. p. 161–172°, with resinification; 2.0 Gm., $[\alpha]_D^{30} = +8.3°$, m. p. completely by 170°; 1.2 Gm., m. p. 172–178°, with resinification; 1.6 Gm., m. p. 205.5–208.5° (dec.). Mixed m. p. of the last crop with cevadine gave no depression. Further purification of several of these crops by recrystallization yielded cevadine.

Similar treatment of fraction A_3^1 yielded additional amounts of cevadine which was purified by recrystallization.

Recrystallization of A_1^1; Isolation of Cevadine and Veratridine.—Forty and seven-tenths grams of fraction A_1^1, $[\alpha]_D^{30} = +6.7°$, was extracted in a Soxhlet extractor with ether for ten hours, at the end of which time it was all in solution. After slow concentration of the amber-colored ether solution to 75 cc. and cooling at 0° had yielded nothing, precipitation of the alkaloids was carried out by the method of Wright and Luff (5). High boiling petroleum ether (Skelly C) was added, with concentration by evaporation to reduce the ether content, until the alkaloids were precipitated from solution. Repetition of this treatment, i.e., solution in ether, addition of Skelly C, and concentration, yielded the crops which are designated, together with their melting points, in Fig. 2.

Several of the fractions obtained in this way, namely, A_1^1-1*c*, A_1^1-1*d*, A_1^1-2*a*, A_1^1-2*c*, upon further recrystallization, yielded pure cevadine.

Fractions A_1^1-1*a* and A_1^1-1*b* were suspected to consist largely of veratridine and the latter was subjected to further purification. Nine and one-half grams of the pale cream-colored A_1^1-1*b*, $[\alpha]_D^{30} = +7.9°$, was dissolved in 45 cc. of 2 *N* H_2SO_4, the solution filtered, and the clear yellow filtrate shaken out with chloroform to remove color. After the dropwise addition of saturated ammonium sulfate solution to the point of distinct turbidity and allowing the mixture to stand for twenty-four hours, the fine needles of alkaloid sulfate were filtered by suction and recrystallized from water. Conversion of the sulfate back to free alkaloid yielded nearly pure veratridine, as indicated by the absorption spectrum;

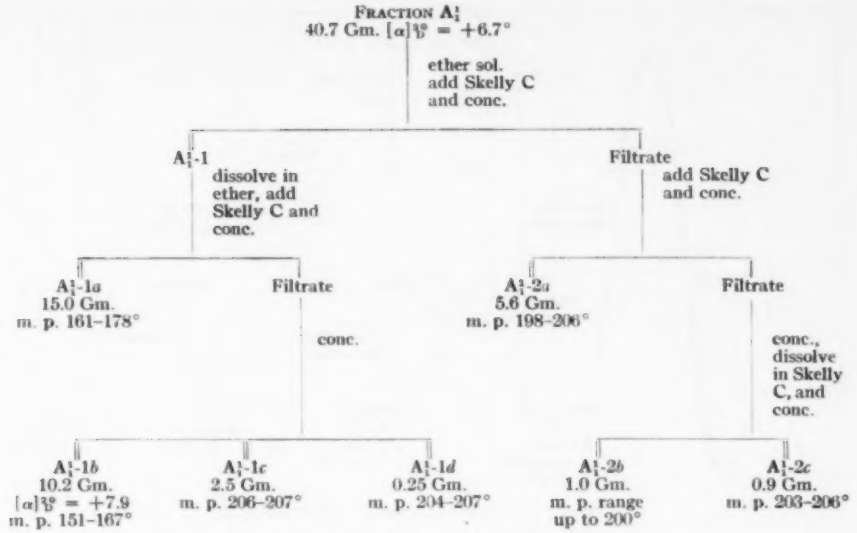

Fig. 2.—Flow sheet of fractional recrystallization of fraction A_1^1.

however, the purest veratridine was obtained from the filtrate of the original veratridine sulfate.

The filtrate from the first sulfate crystals was made cloudy again by the addition of several cc. of saturated ammonium sulfate. After standing for several days in the refrigerator, the white microcrystalline crystals were filtered by suction and dissolved in water. After standing for several days the white needles that separated from this solution were filtered and washed by suction. This veratridine sulfate was suspended in 20 cc. of 5% H_2SO_4 and 50 cc. of chloroform, made alkaline with solid sodium carbonate and ammonia, and extracted with chloroform. The chloroform solution was distilled to dryness *in vacuo* to yield 0.6 Gm. of a white, fluffy residue which sintered at 158–160°, with resinification and melted at 167–184° (dec.). Reconversion of this veratridine to the sulfate, recrystallization of the crystalline sulfate, and conversion back to the free alkaloid caused no change in the absorption spectrum of this sample of veratridine (Fig. 6). It was noted, however, that the alkaloid appeared to be only partially soluble in absolute ether.

No more crystals were obtained from the filtrate of the veratridine sulfate upon further treatment with saturated ammonium sulfate. To precipitate insoluble alkaloid nitrate, 40 cc. of 20% sodium nitrate solution was added. The nitrate was filtered by suction, suspended in acid and chloroform, the free alkaloid liberated by addition of alkali, and extracted with chloroform. The chloroform solution was distilled to dryness *in vacuo* to yield 6.0 Gm. of nearly colorless alkaloid. Attempts to purify this further by conversion to the sulfate were unsuccessful since no insoluble sulfate could be formed. Therefore it was fractionally crystallized from absolute ether. Five crystal crops were obtained and analyzed spectrophotometrically; the first two consisted of mixed crystals of veratridine and cevadine; the third and fourth consisted of nearly pure cevadine, and the fifth and major portion consisted of a mixture of the two alkaloids. The first fraction sintered at 178–181° and appeared to decompose to a brownish red mass. Ultraviolet analysis indicated this fraction to consist of approximately 66% cevadine and 33% veratridine. Upon recrystallization of the second fraction mixed crystals were obtained again; they darkened at 191–192° and were completely resinified at 193°. Ultraviolet analysis (Fig. 9) indicated these crystals to consist of approximately 90% cevadine and 10% veratridine.

Examination of A_1; Evidence for New Alkaloids.—Twenty-five and seven-tenths grams of Fraction A_1, $[\alpha]_D^{25} = 0°$, was extracted in a Soxhlet extractor for twenty-four hours with ether, at the end of which time the extract returning to the pot left only a trace of residue upon evaporation and gave no test with Mayer's reagent. The unextracted residue gave a weak test with Mayer's reagent (A_1-1, 1.2 Gm.). This residue was separated into chloroform-soluble and chloroform-insoluble portions. Attempts to crystallize the latter from various solvents yielded a tan-colored noncrystalline substance, obviously impure, which sintered at 215–217° with darkening and melted at 230–232° (dec.). This melting point is higher than that of either cevadine or veratridine.

The ether extract was treated with Skelly B and concentrated to yield the crops of fractions which are designated, together with their melting points, in Fig. 3. Further attempts at separation of these various fractions by recrystallizations were followed by ultraviolet absorption. Partial separations were accomplished, as indicated by changes in absorption spectra and it appeared from these data that alkaloids other than cevadine and veratridine were

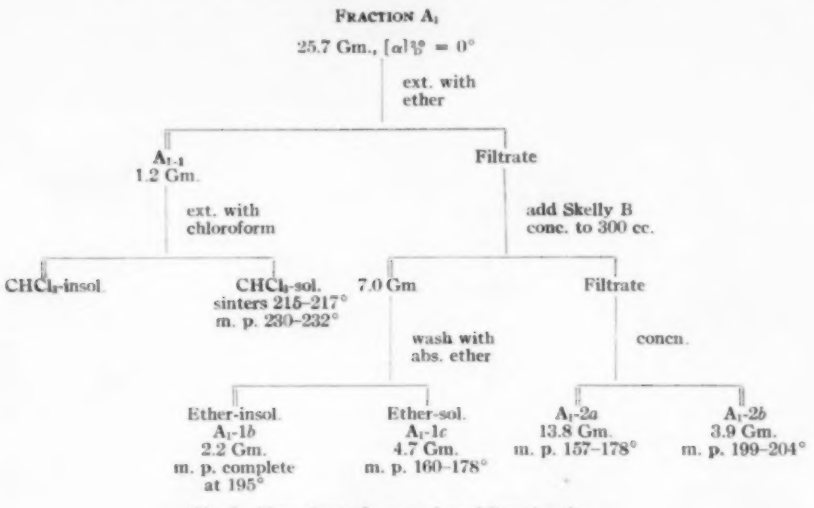

Fig. 3—Flow sheet of separation of Fraction A_1.

present. However, no pure or even relatively pure samples of these were obtained by the procedures used.

To determine the number of components present in Fraction A_1-1*c* and also to study the feasibility of using this procedure for isolation work, this fraction was submitted to chromatographic adsorption studies. Portions of the fraction, in solution, were selectively adsorbed on a column of aluminum oxide, followed by fractional elution with various solvents. Separation of the components was followed by comparing the ratios of the ultraviolet absorption coefficients at certain key wave lengths. Since only trace amounts of alkaloid were present in the eluate fractions, this approach obviated the necessity for precise determination of solution concentrations. From these preliminary studies it appeared that this procedure offers good possibilities, but additional information on the best adsorbents and eluting agents is necessary.

Ultraviolet Absorption of Sabadilla Alkaloids and Related Compounds.—In order to have a basis for interpretation of the spectrophotometric analyses it was necessary to have at hand the absorption spectra of the pure alkaloids, where possible, and of certain related compounds. The absorption spectra of cevadine, veratridine, cevine, commercial sabadilline, veratric acid, and methyl veratrate were determined. Each of these was found to have a characteristic absorption spectrum in the ultraviolet region when the wave length was plotted against the specific extinction coefficient as shown in Figs. 4 to 9.

A Beckmann model DU quartz spectrophotometer was used. For cevadine various solvents, including ethylene chloride, absolute alcohol, and isooctane plus absolute alcohol were used and in each case the general curve was the same. For the others absolute alcohol was used.

An example of how the purification of cevadine was followed by this means is shown in Fig. 5. Curve 1 represents a fairly pure cevadine (m. p. 207–208°) while curve 2 represents the purest sample of cevadine obtained. Figure 4 shows the general curve for pure cevadine while curve 2, Fig. 5, shows the more characteristic portion of the curve.

The veratridine in Fig. 6 was the purest sample obtained in this work. Cevine in Fig. 7 was obtained by hydrolysis of a sample of commercial vera-

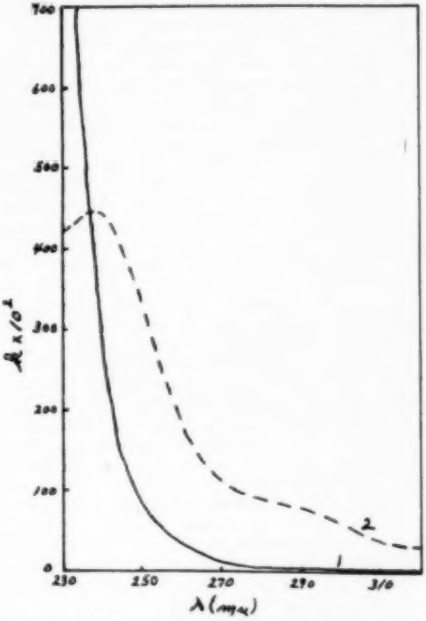

Fig. 4.—Absorption spectra of Cevadine (*1*) and Sabadilline (*2*).

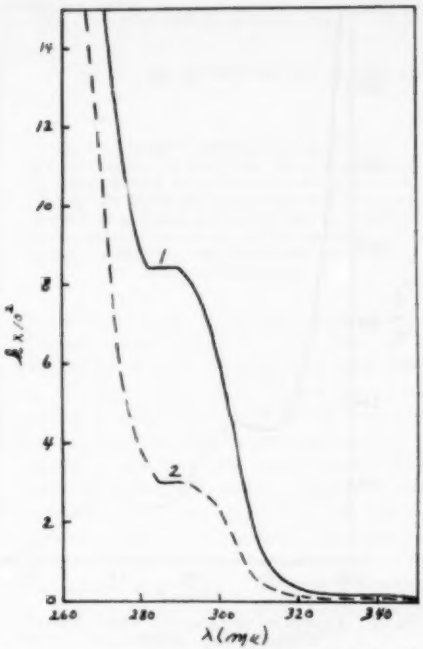

Fig. 5.—Absorption spectra of impure Cevadine (*1*) and pure Cevadine (*2*).

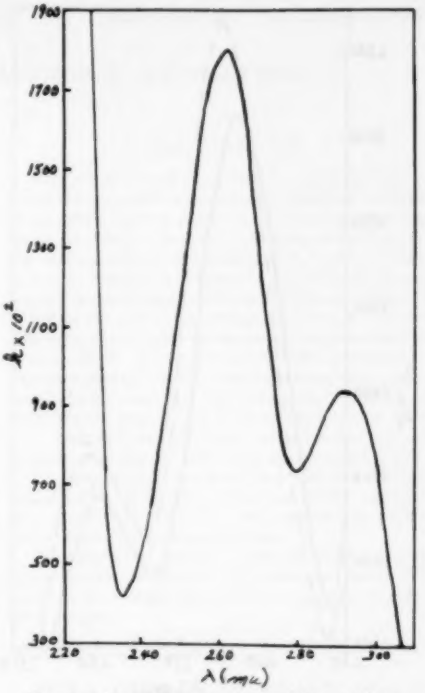

Fig. 6.—Absorption spectrum of Veratridine.

trine followed by several recrystallizations and it is undoubtedly not completely pure. The commercial sabadilline in Fig. 4 was an old sample found in this laboratory and bore a Merck and Co. label. Veratric acid and methyl veratrate in Fig. 8 were synthesized by known procedures[3](6, 7).

DISCUSSION AND SUMMARY

1. It was thought originally that by fractional recrystallization of crude alkaloid Fraction A the cevadine might be separated easily and somewhat selectively from the other alkaloids in the mixture since cevadine is soluble in hot Skelly B. This proved to be true in part, in that cevadine was obtained in nearly pure state in Fraction A_4^1. It was also found, however, that cevadine was present in all of the lower fractions, including A_1 which was insoluble in Skelly B.

2. The greatest amount of pure cevadine was obtained from Fraction A_4^1. The purest cevadine obtained melted higher than that previously reported in the literature, 211–212° (dec.). Recrystallization of this sample from either alcohol or ether had no effect on its purity, as indicated by melting point and absorption spectrum, but the product obtained from alcohol was crystal-

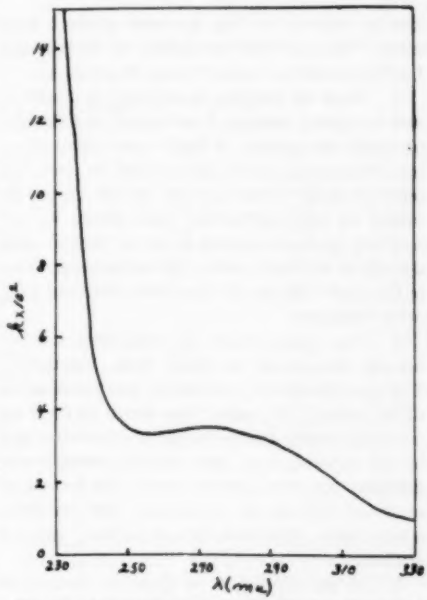

Fig. 7.—Absorption spectrum of Cevine (impure).

[3] Thanks are due Mr. Arnold J. Hennig and Mr. Carl J. Lintner for these two compounds.

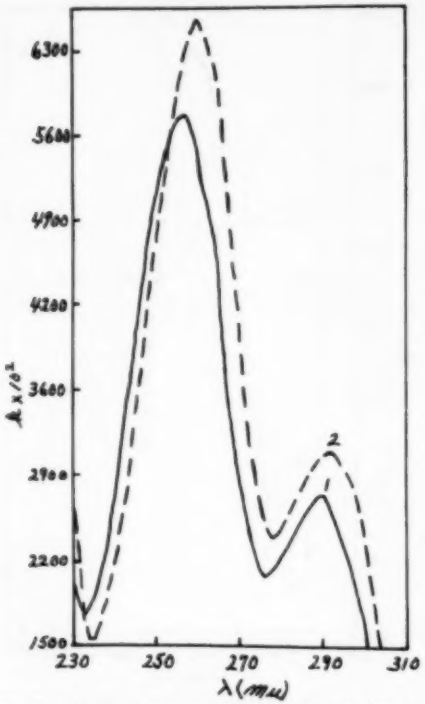

Fig. 8.—Absorption spectra of veratric acid (*1*) and methyl veratrate (*2*).

line in contrast to the granular product from ether. None of the cevadilline of Wright and Luff (5) could be obtained from Fraction A_4^1.

3. From its relative insolubility in Skelly B and its optical rotation Fraction A_2^1 was thought originally to consist of fairly pure veratridine, but attempts at purification failed to yield any pure alkaloid. Some of the crystal crops obtained by recrystallization were shown by absorption spectral analysis to be a veratric acid derivative but their purity did not approach that of the purest sample of veratridine obtained from other fractions.

4. The purest sample of veratridine was obtained, along with cevadine, from Fraction A_1^1. The veratridine was purified by recrystallization of its sulfate. Cevadine was found to form an insoluble nitrate similar to that of veratridine and in the separation of this fraction considerable difficulty was encountered due to the formation of mixed crystals of veratridine and cevadine, which were identified by absorption spectral analysis.

5. In the separation of Fraction A_1 several fractions were obtained in small amounts which

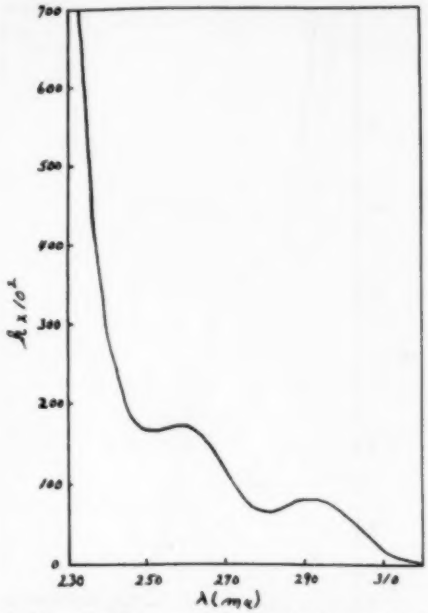

Fig. 9.—Absorption spectrum of mixed crystals of Cevadine and Veratridine.

appeared to be different from any alkaloids previously isolated.

6. The use of optical rotation to follow the fractionation and purification of the alkaloidal mixture was of limited value because of the low rotatory power of the alkaloids and the consequent possibility of large experimental errors. Absorption spectral analysis in the ultraviolet region proved an excellent way of following the purifications. The absorption spectra of the purest samples of cevadine, veratridine, and cevine that were obtained, are reported.

7. Fractional recrystallization procedure, even from a variety of solvents, is not a desirable means of isolating any new alkaloids from the petroleum ether extract of sabadilla seed. Preliminary studies on the selective adsorption and elution of the alkaloids using a chromatographic column and following the separations by means of absorption spectral analysis, appeared promising. This procedure is under further study.

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The Occurrence of Rutin in *Sambucus canadensis**

By BURTON C. KING and ARTHUR E. SCHWARTING

Using an accepted method for its isolation, the occurrence of rutin has been established in elder during a seasonal growing period. The results show that rutin occurs in amounts up to 0.52 per cent and that there is variation in the amount in plants growing under different conditions. Spectrophotometric assay data are presented.

SANDO AND LLOYD (1), in 1924, obtained a pigment, eldrin (rutin), from the flower of elder. These workers demonstrated that eldrin was identical with rutin, obtained from *Eschscholtzia* by preparing derivatives of hydrolytic products and by spectral transmittancy studies.

In the present work rutin was isolated from the leaves and flowers of samples of *Sambucus canadensis* collected in the neighborhood of Lincoln. Extraction studies of the stem and the immature and mature fruit did not reveal the presence of the compound in these parts. Two areas of growth of the plants were used as a source of supply of the leaves and flowers. The one growth (A), was along a water ditch, the other (B), was a roadside growth. Collections of leaves were made at one-week to ten-day intervals from the time of early leaf development until after fruiting. Flowers were collected at immature and mature stages and a commercial sample of elder flowers was also used in the study.

EXPERIMENTAL

About 500 Gm. of fresh shredded material was extracted each time with ethanol in a Soxhlet extractor. The extract was concentrated under reduced pressure to about 200 cc. An equal quantity of distilled water was added and the solution was further concentrated to remove the ethanol. The hot solution was filtered through glass wool and the filtrate allowed to stand in the cold until crystallization was complete. The crop of crystals was collected, weighed, and further purified. This latter process was accomplished by dissolving the crude material in hot absolute ethanol, filtering, and the pigment precipitated by the addition of four volumes of distilled water. The crop collected was dissolved in hot water and the solution was treated with silica gel and then filtered.

Large-scale extractions of the leaves and commercial-dried flowers were carried out on two occasions in a Lloyd Extractor and the product was purified according to the above procedure.

The crude and purified yields are reported in the accompanying table on a moisture-free basis. The samples are designated A and B according to collection source.

Identification of the Rutin

Melting Point.—The melting point data (corrected) for the recrystallized products correspond with rutin. The samples melted indefinitely between 186–192°.

Spectrophotometric Identification.—A spectrophotometric examination (2) of all of the recrystallized products showed a composition of rutin varying between 92.6% and 98.4%. The complete spectrophotometric assay was determined for all samples. A report of No. 10, a leaf product, and No. 11, a flower product, follows:

TABLE I.—RUTIN CONTENT OF ELDER LEAVES AND FLOWERS

No.	Date of Collection	Sample	Moisture, %	Rutin, %	
				Crude	Recrystallized
1	4/24/48	Leaves A	81.8	0.226	0.185
2	5/6/48	Leaves B	80.4	0.046	0.032
3	5/8/48	Leaves A	83.1	0.228	0.180
4	4/15/48	Leaves B	81.5	0.088	0.071
5	5/21/48 (bud stage)	Leaves A	76.7	0.421	0.342
6	5/25/48 (bud stage)	Leaves B	77.6	0.181	0.148
7	6/3/48	Leaves A	77.8	0.431	0.340
8	6/9/48	Leaves B	80.0	0.231	0.184
9	6/14/48 (flower stage)	Leaves A	75.4	0.520	0.442
10	6/21/48 (flower stage)	Leaves B	79.4	0.353	0.288
11	6/21/48	Flowers A	80.5	0.224	0.192
12	6/25/48	Flowers B	82.1	0.182	0.142
13	6/30/48	Leaves A	81.1	0.260	0.200
14	7/7/48	Leaves B	78.9	0.232	0.184
15	7/14/48	Leaves A	81.5	0.345	0.288
16	7/14/48	Leaves B	81.1	0.230	0.210
17		Commercial flower	8.2	0.182	0.154

* Received April 30, 1949, from the Department of Pharmacognosy, College of Phamacy, The University of Nebraska, Lincoln.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

No. 10—$k_{362\text{-}b}$, 32.73; $D_{275\text{-}0}/D_{362\text{-}b}$, 0.876; rutin content, 98.2%.

No. 11—$k_{362\text{-}b}$, 31.13; $D_{275\text{-}0}/D_{362\text{-}b}$, 0.872; rutin content, 94.0%.

Chemical Identification.—The purified glycoside was hydrolyzed with 5% sulfuric acid, the acid being neutralized with barium carbonate. The purified aglycone obtained melted at 313° and an acetyl derivative melted at 196°. The figures correspond with quercetin.

Phenylosazones prepared from the sugar solution were fractionated with acetone (3). The recrystallized fraction soluble in acetone melted at 180°, corresponding to rhamnosazone. The recrystallized acetone-insoluble portion melted at 205°, corresponding to glucosazone.

SUMMARY

Rutin was isolated from the fresh leaves and flowers of *Sambucus canadensis*. The experimental data shows a progressive rise in rutin content during the growing season until the fruiting stage.

The rutin was identified by spectrophotometric and chemical methods.

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1950 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1950 Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1950 Award, nominations must be received on or before January 1, 1950.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its third year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist. Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, will be the recipient of the second award and will give the 1950 Iodine Award lecture.

Book Reviews

Experimental Immunochemistry. By ELVIN A. KABOT and MANFRED M. MAYER. Charles C. Thomas, Springfield, Ill., 1948. xv + 567 pp. 15 x 23 cm. Price $8.75.

There have been very few books written in the field of immunochemistry, despite the fluorishing and profitable research in the field. Some of the best earlier books are now out of print.

Although this book by Kabot and Mayer is not complete, it brings together, for the first time, the scattered techniques used in immunochemistry. These methods, which are drawn from many fields, are described in working detail and discussed as to both applicability and limitations.

During this era of radioactive isotope tracers, the fact is apt to be overlooked that the immunochemist has a large store of tagged molecules at his disposal and these tagged molecules are able to yield precise measurements of the magnitude of those of the atomic physicist. In fact, the authors stress these analytical relationships, bringing out the important functions they perform.

The book is divided into four main parts. Part I deals with methodology and is concerned with general reactions. Part II gives details of the applications of these methods. Part III is concerned with actual working instructions of the many techniques used. Part IV gives methods of preparing individual proteins, enzymes and their precursors, antibodies, and other biochemical entities used in this field. An appendix gives detail of many common techniques which may not be familiar to the uninitiated in this field.

The authors are to be congratulated for bringing this material together in one volume and for presenting it in an interesting and stimulating manner. The addition of eighty tables and eighty figures makes the book more useful.

The Plant Alkaloids. 4th ed. By THOMAS A. HENRY. The Blakiston Co., Philadelphia, 1949. xxiii + 804 pp. 15 x 23.5 cm. Price $12.

Like many other branches of chemistry, alkaloid chemistry has progressed in rapid strides during the past decade. Hence it is fitting that this standard reference work should be thoroughly revised at this time.

The fourth edition of this useful reference follows in general the pattern of previous editions with certain convenient modifications. Because either strictly chemical or strictly botanical classifications often obscure physiological relationships, the chemical classification has been modified in cases where an extensive series, including several chemical types, occurs in one plant or in closely related plants. If this results in a chemical group being recorded in more than one place, the author has been generous with his cross references. Little has been said concerning the modern nomenclature based on electronic configurations.

A Handbook of Commonly Used Drugs. By MICHEL PIJOAN and CLARK H. YEAGER. Charles C. Thomas, Springfield, Ill., 1947. 198 pp. 15 x 23 cm. Price $3.75.

The authors of this small handbook have accumulated the basic facts on the use of common drugs for use on field expeditions when extensive reference material is difficult to obtain. The authors have done an unusually good job in condensing essential information in so few pages. Although brief, there has been no sacrifice in accuracy. In addition, a very selective bibliography is presented which is a key to review literature and highly significant papers. This book should appeal to those needing hand-books of this type.

The Chemistry and Technology of Enzymes. By HENRY TAUBER. John Wiley and Sons, New York, 1949. viii + 550 pp. 15 x 23 cm. Price $7.50.

The Chemistry and Technology of Enzymes is an expansion of a previous book, *Enzyme Technology*, by the same author.

The book is divided, almost equally, into two portions. Part I deals with the chemistry of the enzymes and Part II, the technology.

In the first part of the book will be found an introductory chapter which gives principles of nomenclature and classification, activity, *p*H curves, principles of enzyme kinetics, including a discussion of the Michaelis-Menten theory, enzyme activation and inhibition, general methods of preparing enzymes and other general consideration. This is followed by chapters on individual classes of enzymes, i.e., esterases, carbohydrases, phosphatases, etc.

Part II is characterizied by chapters on the role of enzymes in industry. Yeast, alcohol production, brewing, mold and bacterial fermentations, and many other industrial applications are discussed. Included is an important chapter on microbiological methods for estimating the amino acids and vitamins.

Each chapter contains many well-selected references to the more pertinent papers in the field.

Workers in the enzyme and related fields will find this book a useful and modern handbook.

Pharmaceutical Arithmetic. By IGNATIUS J. BELLAFIORE. 2nd ed. C. V. Mosby Co., St. Louis, Mo., 1947. 393 pp. 19 x 27 cm. Price $3.75.

The second edition of this workbook in pharmaceutical calculations essentially follows the style of the first edition. Some new illustrations, a new chapter on the preparation of isotonic solutions, and material on the relationship between density and temperature have been added. The pages may be removed easily for grading by the instructor. The book is intended for use in the college, but some pharmacists will find it interesting, particularly if they enjoy working problems.

Vernalization and Photoperiodism, A Symposium. By A. E. MURNEEK and R. O. WHYTE. Waltham, Massachusetts: The Chronica Botanica Company; New York City: Stechert-Hafner, Incorporated, 1948. 196 pp. 10.5 x 7.5 cm. Price $4.50.

Causes of flower formation are factors of great current interest in phases of plant growth studies. Vernalization, which is concerned with a temperature gradation such as the effects of cold upon seed germination, and photoperiodism, as it deals with the effects of varying day lengths, are two fields of study from which one seeks possible explanations of why plants flower. Murneek and Whyte's *Symposium* makes use of collaborations from other experts and gives an outstanding review of the major concepts from the fields of vernalization and photoperiodism. The text does not give the final explanation of flower formation, but rather brings together leading researches which stimulate the student to his fullest conjecture.

References are abundant. There are eleven discussions and three special supplements, plus numerous tables and plates. Included is an interesting scientific discussion of the "controversial" aspect of Lysenko's findings. Generally the *Symposium* is directed to concepts of economic significance in agriculture. However, frequent use of certain important drug plant material renders it of considerable value to the pharmacognosist as well.—H. W. YOUNGKEN, JR.

Injury and Death of Bacteria by Chemical Agents. By OTTO RAHN. Biodynamica, Normandy, Mo., 1945. 183 pp. 17 x 24 cm. Price $4.50.

The problem of the cause and mechanism of death of bacteria in the presence of chemicals (germicides) has stimulated bacteriologists and biochemists for many decades. Dr. Rahn has not answered all of the questions in this area, but his discussion of the "logarithmic order of death" is a helpful and stimulating approach.

Although there are many who do not agree that survival curves of bacteria are actually exponential, such biologists will have to examine their concepts more critically in the light of the author's arguments.

Following the development of the arguments for the logarithmic order of death (Part 1), the author uses these concepts to show the action of disinfectants and antiseptics (the author's term for bacteriostatic agents).

The author shows that the efficiency of disinfectants increases with concentration, but not in the same way with all disinfectants. The phenol coefficient as a standard is criticized because phenol action changes with concentration in a special manner. A new method is proposed which gives the death times for any concentration of germicide directly rather than in comparative terms and permits the quantitative measure of the interference of organic matter.

Although many methods for measuring the interference with bacterial growth and reproduction by drugs have been developed, no single method has yet yielded completely satisfactory answers. Professor Rahn has given many stimulating ideas as points of departure for future studies.

Opiate Addiction. By ALFRED R. LINDESMITH. Principia Press, Bloomington, Ind., 1947. lx + 235 pp. 15 x 23.5 cm. Price $3.

Opiate addiction is one of the oldest sociologic problems mankind has had to face. During more recent years a number of different approaches have been made. Attempts have been made to synthesize compounds capable of providing a level of analgesia comparable to that provided by morphine without the attending addicting properties. Others have tried to determine what happens physiologically and pathologically to the tissues of the addict, while still others have used a psychiatric approach.

In a sociologic approach, Dr. Lindesmith points out that both current police practices and medical practice of handling addiction are dependent on both punishment and medication as methods of cure. The importance of the difference between habitation and addition has been stressed by the author, who believes that the crux of the matter lies in the awareness by the addict that the withdrawal symptoms can be alleviated by the use of drugs.

In an area so difficult and complex as opiate addiction, certainly many will take issue with Dr. Lindesmith on some of his findings, but all will acknowledge that it represents a skillful example of social inquiry.

Collecteana Pharmaceutica Suecica. Edited by KUNGL. Farmaceutiska Institutets Bibliotek Stockholm. Volume I, 1946, Volume II, 1947. Pagination irregular. 15 x 22.5 cm. Ivar Haeggströms, Stockholm, Publishers.

Collecteana Pharmaceutica Suecica is a new annual publication of the Library of the Royal Institute of Pharmacy (Sweden) begun in 1947, covering the year 1946. The collection consists of separate impressions from Swedish pharmaceutical periodicals and is written or summarized in one of the principal scientific languages. References to the original journal in which the publication appeared remain in the text for convenience. Each contribution is separately paged. The majority of the papers are concerned with analytical methods, although some deal with other pharmaceutical sciences such as pharmacology, toxicology, pharmacognosy, and organic chemistry.

Laboratory Guide in Pharmacology for Pharmacy and Dental Students. By HARALD G. O. HOLCK. Burgess Publishing Co., Minneapolis, Minn., 1949. xiii + 114 pp. 21 x 27 cm. Price $2.75.

Teachers in colleges of pharmacy and dentistry will welcome this well-conceived and well-executed laboratory guide in pharmacology. The sequence of experiments is well planned and each individual experiment is developed so as to put across a pharmacological principle with the simplest possible physiological technique. By concentrating on pharmacological principle rather than use, the manual is equally adaptable to students of pharmacy and dentistry. The drawings are very clear and all directions are easily understood. Introductory experiments in "pharmacy" are given for the dental students.

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